5

0   PROTEIN TYROSINE KINASES

                   BACKGROUND OF THE INVENTION

.5   FIELD OF THE INVENTION
         This application relates to novel protein tyrosine kinases,
     the   nucleic   acid   sequences   encoding   these   proteins,   the
     extracellular domains of the proteins, ligands to the protein
     tyrosine kinases, antibodies specific for the encoded proteins and
!0   methods of use therefor. In particular, this application relates to
     the novel receptor protein tyrosine kinases designated Rse and
     HPTK6.

                   DESCRIPTION OF RELATED ART
!5   Intracellular   signals   which   control   cell   growth   and
     differentiation are often mediated by tyrosine kinase proteins.
     Tyrosine kinases catalyze protein phosphorylation using tyrosine as
     a substrate for phosphorylation. Members of the tyrosine kinase
     family can be recognized by the presence of several conserved amino
30   acid regions in the tyrosine kinase catalytic domain (Hanks et al.,
     Science: 241: 42-52 [1988]). The tyrosine kinase domain is crucial
     for the signal transduction pathways required for mitogenesis,
     transformation and cell differentiation. Certain tyrosine kinases
     predominantly stimulate cell growth and differentiation, whereas
35   other tyrosine kinases arrest growth and promote differentiation.
     Furthermore, depending on the cellular environment in which it is

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expressed, the same tyrosine kinase may either stimulate, or inhibit, cell proliferation (Schlessinger et al., Neuron, 9: 383-391 [1992]).

5   Tyrosine kinase proteins can be classified as either receptor tyrosine kinases or intracellular tyrosine kinases. Receptor tyrosine kinases (rPTKs) convey extracellular signals to intracellular signaling pathways thereby controlling cell proliferation and differentiation. These rPTKs share a similar architecture, with an intracellular catalytic portion, a
10   transmembrane domain and an extracellular ligand-binding domain. (Schesslinger et al., supra). The extracellular domains (ECDs), which are responsible for ligand binding and transmission of biological signals, have been shown to be composed of a number of distinct structural motifs. The intracellular domain comprises a
15   catalytic protein tyrosine kinase. The binding of ligand to the extracellular portion is believed to promote dimerization of the rPTK resulting in transphosphorylation and activation of the intracellular tyrosine kinase domain. In addition to their catalytic function, the intracellular domains (ICDs) of rPTKs may
20   also serve as binding sites for other components of the signal transduction pathway. In particular, some proteins containing src-homology 2 (SH2) domains have been shown to interact in a phosphorylation-dependent and sequence specific manner to specific tyrosine residues within the ICD (Cantley et al., Cell, 64: 281-302
25   [1991]).

A large number of protein tyrosine kinases have been characterized on the basis of their amino acid and nucleic acid sequences. For a review of these proteins see Hanks et al., supra.

WO 93/15201 discloses isolation of several novel rPTK genes
30   found in human megakaryocytic and lymphocytic cells using degenerate oligonucleotide probes as primers in a polymerase chain reaction (PCR) to amplify tyrosine kinase DNA segments.

The recent publication by Johnson et al., Proc. Natl. Acad.

Sci., 90: 5677-5681 (1993) discusses the characterization of a receptor tyrosine kinase called discoidin domain receptor (i.e., DDR) which is abundantly expressed in breast carcinoma cell lines. DDR is considered to have two features not found in other receptor tyrosine kinases. First, a region of the amino acid sequence near the N terminus of DDR contains a "discoidin I-like domain". This determination was based on the sequence identity between this region and the protein, discoidin I (see Figure 5 of Johnson et al.). Discoidin I-like domains are present as tandem repeats at the C terminus of the light chains of factor V (Kane, W.H. & Davie, E.W., Proc. Natl. Acad. Sci., 83: 6800-6804 [1986]), factor VIII (Toole et al., Nature(London), 312: 342-347 [1984]) and Vehar et al., Nature(London), 312: 337-342 [1984], and two milk fat globule membrane proteins, MFG.E8 (see Stubbs et al., Proc. Natl. Acad. Sci., 87: 8417-8421 [1991]) and BA46 (see Larocca et al., Cancer Res., 51: 4994-4998 [1991]). Second, the DDR protein has an extensive proline/glycine-rich region between the discoidin I-like domain and the transmembrane domain and another such region between the transmembrane domain and the C-terminal tyrosine kinase domain. These proline/glycine-rich regions are not found in other receptor protein tyrosine kinases. The catalytic domain of DDR shares 45% sequence identity with the trk protein catalytic domain disclosed in Martin-Zanca et al., Mol. Cell. Biol., 9:24-33 (1989). Zerlin et al. disclose isolation of the murine equivalent of the DDR rPTK found by Johnson et al., which they call NEP (Oncogene, 8: 2731-2939 [1993]).

WO 92/14748 discloses a receptor, designated KDR, which is classified as a type III receptor tyrosine kinase and binds to vascular endothelial cell growth factor. The type III group of rPTKs includes the c-kit proto-oncogene and the receptors for platelet derived growth factor (PDGF) and colony stimulating factor-1 (CSF-1).

Matthews et al., Proc. Natl. Acad. Sci., 88: 9026-9030 (1991)

refer to the isolation of rPTK clone from a population of hematopoietic murine cells which, like KDR, exhibits a close sequence identity to *c-kit*. This receptor is called *flk-1*. The *flk-1* receptor was isolated using an anti-sense oligonucleotide

5   primer and anchored PCR. Chromosomal mapping indicated that the *flk-1*, *kit* and *pdgfra* genes are closely linked. Matthews et al., Cell, 65: 1143-1152 (1991) discuss isolation of a rPTK called *flk-2*, from stem cell-enriched murine hematopoietic tissue. US Patent No. 5,185,438 also refers to the rPTKs, flk-1 and flk-2, which are

10   said to be expressed in primitive hematopoietic cells but not in mature hematopoietic cells.

     Lai et al., Neuron, 6: 691-704 (1991) used PCR to identify several cDNAs encoding part of the tyrosine kinase domains of various rat rPTKs. The newly isolated sequences were designated

15   *tyro-1 to tyro-13*. Because preferential expression of several of the sequences in the developing vertebrate nervous system was evidenced, Lai et al. concluded that protein-tyrosine kinases appear to play a central role in neural development.

     Holtrich et al., Proc. Natl. Acad. Sci., 88:10411-10415 (1991)

20   studied the expression of protein-tyrosine kinases in normal human lung and tumor cells by PCR followed by molecular cloning and sequence analysis. Six known PTKs (*yes*, *fgr*, *lyn*, *hck*, *pdgfb-r* and *csf1-r*) were detected as well as two new PTKs. One of the proteins detected appeared to be cytosolic. The other PTK, designated *TKF*,

25   was found to be related to fibroblast growth factor receptor and was only found expressed in the lung.

     WO 93/14124 discloses the cloning, sequencing and expression of a human rPTK termed *tie* which is expressed in cultured endothelial cells as well as tumor cell lines. The extracellular

30   domain (ECD) of *tie* was found to contain stretches of amino acid sequence having features of the immunoglobulin, epidermal growth factor and fibronectin type III repeat protein families.

     Partanen et al., Proc. Natl. Acad. Sci., 87: 8913-8917 (1990)

analyzed PCR amplified cDNA clones which lead to the identification of 14 different tyrosine kinase-related sequences, designated *JTK1-14*. Based on the pattern of expression of the clones, it was suggested that the tyrosine kinases encoded by the complete
5   sequences most probably play a role in the differentiation of megakaryoblasts or in the physiology of platelets.

       While Partanen *et al.* discuss isolation of the partial JTK11 cDNA clone, the later publication by Janssen *et al.*, Oncogene, 6: 2113-2120 (1991), reports the cDNA cloning of the entire oncogene
10   (designated *UFO*) encoding a 894 amino acid polypeptide. Janssen *et al.* identified the *UFO* tyrosine kinase receptor by DNA transfection analysis of bone marrow cells from a patient suffering from a chronic myeloproliferative disorder. It is noted in this publication that several oncogene products are rPTKs, *e.g.* colony-
15   stimulating factor-1 and *TRK*. Around the same time that Janssen *et al.* isolated the rPTK they call *UFO*, O'Bryan *et al.* isolated the same rPTK (which they designate *Axl*) from human myeloid leukemia cells (O'Bryan *et al.*, Mol. Cell. Biol., 11: 5016-5031 [1991]). *Axl* is a transforming gene which encodes a rPTK having two fibronectin
20   type III repeats and two immunoglobulin-like repeats in the extracellular domain thereof. These motifs are also found in the extracellular domain of the receptor-like protein tyrosine phosphatase, PTPμ (Brady-Kalnay *et al.*, J. Cell Biol., 122: 961-972 [1993]). The immunoglobulin domain and four fibronectin type-III
25   repeats of PTPμ are similar to the motifs found in cell-cell adhesion molecules. Brady-Kalnay *et al.* propose that the ligand for the PTPμ may be another PTPμ on an adjacent cell.

       Faust *et al.*, Oncogene, 7: 1287-1293 (1992) disclose cloning of the mouse homologue of the *UFO* oncogene identified in the
30   publication by Janssen *et al.* This murine tyrosine kinase has an overall sequence identity of 87.6% with the human sequence. The extracellular domain of the *UFO* receptor is characterized by the existence of two immunoglobulin-like (IgL) and two fibronectin type

III (FNIII) repeats. As discussed in Faust *et al.*, a combination of IgL and FNIII domains are also found in several neural cell adhesion molecules and receptor tyrosine phosphatases suggesting that these structures are important for intercellular communication.

Wilks *et al.*, Gene, 85: 67-74 (1989) used degenerate oligo-deoxyribonucleotide (oligo) primers derived from amino acid sequence motifs held in common between all members of the PTK family to prime the amplification of PTK sequences. It was found that the most effective type of primer for identification of PTK sequences is a short, moderately degenerate, oligo primer. Using the techniques disclosed, Wilks and his co-workers isolated a new mammalian PTK sequence as well as other known PTK sequences.

Bräuninger *et al.*, Gene, 110(2): 205-211 (1992) disclose isolation of a human gene encoding an intracellular protein belonging to a new subclass of protein tyrosine kinases. The clone, designated *csk*, was found to be expressed in human lung and macrophages. The *csk* gene was distinguished from the *src* family of proto-oncogenes by the lack of certain tyrosine autophosphorylation sites in the amino acid sequence and the lack of a N-terminal myristylation site.

It is evident that a number of rPTKs are involved in cell growth and differentiation, many of which have been characterized to date.

Additional rPTKs are needed in order to further study growth and differentiation of cells, for use as therapeutic agents and for diagnostic use.

Accordingly, it is an object of this invention to identify and purify one or more novel protein tyrosine kinase receptors. It is yet another object to provide derivatives and modified forms of such new polypeptides, including amino acid sequence variants and covalent derivatives thereof.

It is another object to provide nucleic acid encoding such

novel rPTKs and to use this nucleic acid to produce rPTKs in recombinant cell culture. The rPTK protein thus produced can be used for investigational, therapeutic or diagnostic use. Nucleic acid sequences which hybridize with the DNA or RNA encoding the

5 proteins described herein can also be used as anti-sense oligonucleotides to inhibit protein tyrosine kinase activity either *in vivo* or *in vitro*.

It is a further object to provide amino acid sequences encoding the ECDs of the novel rPTKs, which sequences are useful

10 for *in vitro* assays or for use as therapeutic agents. The ECDs, or variants thereof, can also be used as immunogens for raising antibodies, including agonist antibodies to the rPTKs. Nucleic acid sequences encoding the novel rPTK ECDs are needed in order to make these polypeptides recombinantly.

15 Ligands to the novel rPTKs are also desirable for use as therapeutic agents to stimulate the receptor and thereby stimulate cell growth and/or differentiation. Such ligands are useful for determining the function and biological activity of the receptors.

These and other objects will be apparent to the ordinary

20 artisan upon consideration of the specification as a whole.

SUMMARY OF THE INVENTION

These objects are accomplished, in one aspect, by providing isolated Rse or HPTK6 rPTKs that may be antigenically or

25 biologically active.

In another aspect, the invention provides a composition comprising biologically active Rse or HPTK6 and a pharmaceutically acceptable carrier.

According to another object of the invention, the isolated

30 extracellular domains of each of the novel rPTKs are provided which can be used to raise antibodies against each of the novel rPTKs.

In another aspect, the invention provides isolated ligands which bind to the extracellular domain of the rPTKs. Such ligands

can act as antagonists or agonists and thereby either stimulate, or inhibit, tyrosine kinase activity of the rPTKs.

The invention also provides isolated nucleic acid sequences encoding the entire rPTK amino acid sequence or the extracellular domain thereof, as well as nucleic acid sequences encoding protein ligands to the novel rPTK proteins.

In still further aspects, the nucleic acid is provided in a replicable vector comprising the nucleic acid encoding the proteins disclosed. The invention also provides host cells transformed with the vector. A method of using the nucleic acid encoding the proteins to effect the production of the novel proteins is also provided which comprises expressing the nucleic acid in a culture of the transformed host cells and recovering the protein from the host cell culture.

In further embodiments, the invention provides a method of enhancing cell growth or differentiation comprising administering to a mammalian patient in need of such treatment an exogenous compound selected from the group consisting of: Rse rPTK; HPTK6 rPTK; agonist ligand to Rse rPTK; and agonist ligand to HPTK6 rPTK, in an amount effective in inducing cell growth or differentiation.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1A depicts the nucleic acid sequence for human Rse (SEQ ID NO: 1) and the deduced amino acid sequence thereof (SEQ ID NO: 2). The nucleic acid sequence of the extracellular domain of Rse (SEQ ID NO: 5) and the amino acid sequence of the extracellular domain of Rse (SEQ ID NO: 6) are indicated by dots. Figure 1B depicts the nucleic acid sequence for murine Rse (SEQ ID NO: 9) and the deduced amino acid sequence thereof (SEQ ID NO: 10). The nucleic acid sequence of the extracellular domain (SEQ ID NO: 11) and the amino acid sequence of the extracellular domain thereof (SEQ ID NO: 12) are indicated by dots.

In Figures 1A and 1B, the composite nucleic acid sequence

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determined from overlapping cDNA clones is shown on the bottom line. The translated sequence, in single-letter amino acid code, is shown on the top line. The predicted signal sequences are printed in bold-type, and the potential sites for N-linked glycosylation are indicated with an (*). The putative transmembrane domains are boxed. The arrows delineate the start and end of the putative tyrosine kinase domain, and within that domain, the consensus sites for Mg^{2+}-ATP binding (beginning at amino acids 525 and 515 of the human and murine Rse proteins, respectively) and the region often used to predict substrate specificity (beginning at amino acids 652 and 642 of the human and murine Rse proteins, respectively) are underlined. Human and murine Rse sequences represent a total of 3,611 and 3,785 nucleotides, respectively, determined from overlapping clones sequenced in both directions. The human Rse cDNA sequence ends at an internal EcoRI site in the 3' untranslated region; the murine Rse cDNA includes the polyadenylation sequence.

Figure 2 depicts the nucleic acid sequence for human HPTK6 (SEQ ID NO: 3) and the deduced amino acid sequence thereof (SEQ ID NO: 4). The nucleic acid sequence of the extracellular domain of HPTK6 (SEQ ID NO: 7) and the amino acid sequence of the extracellular domain of HPTK6 (SEQ ID NO: 8) are in bold, the putative transmembrane domain is boxed, the amino acid residues forming the signal sequence are indicated with an (*) and the putative ATP binding site in the kinase domain is indicated by dots. The arrows delineate the start and end of the putative tyrosine kinase domain.

Figure 3 is a diagrammatic representation of the structural domains and hydrophobicity plot of human and murine Rse. A schematic representation of the immunoglobulin-like (IgL) domains, fibronectin type III domains (FNIII), transmembrane domain (TM) and tyrosine kinase (Kinase) domains of Rse is shown on the top line. Below, the hydrophobicity profile of human Rse and murine Rse is shown. The HYDRO program (Genentech, Inc.) was used to obtain the

hydrophobicity plots.

Figure 4 depicts a comparison of the amino acid sequences of human and murine Rse (i.e., hRSE and mRSE, SEQ ID NOS: 2 and 10, respectively), and Axl (i.e., hAXL and mAXL, SEQ ID NOS: 34 and 35, respectively). Sequences were aligned using the ALIGN program. Gaps introduced for optimal alignment are indicated by dots. The amino acid positions are numbered from the initiation methionine. Conserved residues are boxed. Immunoglobulin-like domains 1 and 2 (IgL-1 and IgL-2), fibronectin type III-like domains 1 and 2 (FN-1 and FN-2), and the tyrosine kinase homology region are indicated. The highly conserved amino acids in the IgL domains are indicated by (*), and the eleven highly conserved domains (Hanks et al., supra) within the tyrosine kinase region are marked.

Figure 5 illustrates expression and activation of gD-Rse. Total lysates from NIH3T3 cells (lanes 1, 3, 5, 7, 9, and 11) or 3T3.gD.R11 cells (lanes 2, 4, 6, 8, 10, and 12) were immunoprecipitated with the antibody 5B6 which detects the gD portion of the fusion protein, and the immunoprecipitates were resolved by SDS-PAGE and immunoblotted with anti-phosphotyrosine antibodies (lanes 7-12). After the blots were developed, they were stripped and re-probed with antibody 5B6 (lanes 1-6). Lysates were prepared from cells grown in the absence of added antibody (lanes 1, 2, 7, and 8) or incubated with antibody 5B6 (lanes 3, 4, 9, and 10) or an isotype-matched control antibody A3.1.2 (lanes 5, 6, 11, and 12). Molecular masses (kDa) are indicated on the right.

Figure 6 depicts a time course of antibody induced stimulation of gD-Rse tyrosine kinase activity. 3T3.gD.R11 cells were incubated without (-) or with antibody 5B6 for 10, 30, 60, or 120 minutes (Min.) as indicated. Western blots were prepared as described for Figure 5. The blot was reacted first with the anti-phosphotyrosine antibody 5E2 (α-pTyr) then stripped and reacted with antibody 5B6 (α-gD) to control for the amount of gD-Rse on the blots.

Figures 7A and 7B show a Northern blot analysis of Rse mRNA

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expression in adult human tissues. In Figure 7A, a Northern blot containing 2 μg of poly(A) RNA isolated from human tissues was hybridized to a ^{32}P-labeled probe corresponding to human Rse nucleotides 195-680 (Figure 1A). Positions of markers are indicated on the right in Kb. Lane 1: heart, lane 2: brain, lane 3: placenta, lane 4: lung, lane 5: liver, lane 6: skeletal muscle, lane 7: kidney, lane 8: pancreas. In Figure 7B, the blot shown in Figure 7A was washed and then hybridized with a ^{32}P-labeled beta-actin probe to confirm the integrity of the RNA samples.

Figures 8A and 8B depict the chromosomal localization of the human Rse gene. Figure 8A depicts the ethidium-stained PCR product of one of two amplifications using independent primer sets (Btk 3-1^{2724}, Btk 3-4), corresponding to unique 3'-untranslated sequences in Rse amplifying genomic DNA derived from a panel of human-CHO hybrid cell lines (lanes 1-25), human control (lane 26) or hamster control (lane 27). In Figure 8B, the matrix of hybrid cell line number and corresponding human chromosome is highlighted to indicate the match of the PCR product with human chromosome 15.

Figure 9 depicts stimulation of gD-Rse by polyclonal antibodies. Immunoprecipitates from control NIH3T3 cells (lanes 1 and 3) or 3T3.gD.R11 cells (2 and 4) were prepared using the anti-gD antibody 5B6, resolved by SDS-PAGE and immunoblotted with antiphosphotyrosine antibodies. Cells were either untreated (lanes 1 and 2) or treated (lanes 3 and 4) for 10 minutes with rabbit polyclonal antiserum prepared against a fusion protein containing the extracellular domain of Rse.

Figures 10A and 10B show a Northern blot analysis of HPTK6 mRNA expression in adult (Figure 10A) and fetal (Figure 10B) human tissues. A Northern blot containing 2 μg of poly(A) RNA isolated from human tissues was hybridized to a ^{32}P-labeled probe corresponding to human HPTK6 nucleotides 11-622 (Figure 2). Positions of markers are indicated on the left in Kb.

Figures 11A and 11B show a Northern blot analysis of HPTK6

mRNA expression in adult mouse tissue. In Figure 11A, a Northern
blot containing 2 μg of poly(A) RNA isolated from human tissues was
hybridized to a ^{32}P-labeled probe corresponding to human HPTK6
nucleotides 11-622 (Figure 2). Positions of markers are indicated
on the left in Kb. For·Figure 11B, the blot shown in Figure 11A
was washed and then hybridized with a ^{32}P-labeled beta-actin probe
to confirm the integrity of the RNA samples.

Figures 12A, 12B, and 12C depict *in situ* hybridization of
HPTK6 in human (Figure 12A) and mouse (Figures 12B and 12C) fetal
tissue. Transverse section through human or mouse embryos were
hybridized with ^{32}P-labeled antisense (-ve) and sense (tve) strands.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

I. Definitions

In general, the following words or phrases have the indicated
definition when used in the description, examples, and claims:

"Receptor Protein Tyrosine Kinases" (rPTKs), when used
throughout the detailed description of the invention, refers to Rse
and HPTK6 proteins. It also refers to both full-sequence and ECD
unless specifically stated otherwise.

"Rse" is defined herein to be any polypeptide sequence that
possesses a biological property of a naturally occurring
polypeptide comprising the polypeptide sequence of Figure 1A or 1B.

"HPTK6" is defined herein to be any polypeptide sequence that
possesses a biological property of a naturally occurring
polypeptide comprising the polypeptide sequence of Figure 2.

"Biological property" for the purposes herein means an *in vivo*
effector or antigenic function or activity that is directly or
indirectly performed by Rse or HPTK6 (whether in its native or
denatured conformation). Effector functions include receptor
function, ligand binding, signal transduction, phosphorylation

using tyrosine as a substrate for phosphorylation, dimerization of the rPTK resulting in transphosphorylation and activation of the catalytic kinase domain, any enzyme activity or enzyme modulatory activity (e.g., tyrosine kinase activity), stimulation of cell

5 growth and/or differentiation, inhibition of cell growth or proliferation, or any structural role. However, effector functions do not include possession of an epitope or antigenic site that is capable of cross-reacting with antibodies raised against Rse or HPTK6. An antigenic function means possession of an epitope or

10 antigenic site that is capable of cross-reacting with antibodies raised against the polypeptide sequence of a naturally occurring polypeptide comprising the polypeptide sequence of Figure 1A, 1B or Figure 2.

"Biologically active" rPTK is defined herein as a polypeptide

15 that shares an effector function of rPTK and that may (but need not) in addition possess an antigenic function. A principal known effector function of rPTK is its ability to catalyze protein phosphorylation using tyrosine as a substrate for phosphorylation. The biological activity of rPTK may be further characterized by its

20 ability to stimulate cell growth or differentiation *in vivo* or *in vitro*.

"Antigenically active" rPTK is defined as a polypeptide that possesses an antigenic function of rPTK and that may (but need not) in addition possess an effector function.

25 In preferred embodiments, antigenically active rPTK is a polypeptide that binds with an affinity of at least about 10^6 1/mole to an antibody capable of binding rPTK. Ordinarily, the polypeptide binds with an affinity of at least about 10^7 1/mole. Isolated antibody capable of binding rPTK is an antibody that is

30 identified and separated from a component of the natural environment in which it may be present. Most preferably, the antigenically active rPTK is a polypeptide that binds to an antibody capable of binding rPTK in its native conformation. rPTK

in its native conformation is rPTK as found in nature that has not been denatured by chaotropic agents, heat, or other treatment that substantially modifies the three-dimensional structure of rPTK as determined, for example, by migration on non-reducing, non-

5   denaturing sizing gels. Antibody used in this determination is rabbit polyclonal antibody raised by formulating native rPTK from a non-rabbit species in Freund's complete adjuvant, subcutaneously injecting the formulation, and boosting the immune response by intraperitoneal injection of the formulation until the titer of

10 anti-rPTK antibody plateaus.

Ordinarily, biologically or antigenically active rPTK will have an amino acid sequence having at least 75% amino acid sequence identity with the mature rPTK amino acid sequence shown in either Figure 1A, 1B or Figure 2, more preferably at least 80%, more

15 preferably at least 85%, more preferably at least 90%, and most preferably at least 95%. Identity or homology with respect to this sequence is defined herein as the percentage of amino acid residues in the candidate sequence that are identical with the rPTK residues, after aligning the sequences and introducing gaps, if

20 necessary, to achieve the maximum percent sequence identity, and not considering any conservative substitutions as part of the sequence identity. None of N-terminal, C-terminal, or internal extensions, deletions, or insertions into the rPTK sequence shall be construed as affecting sequence identity or homology.

25 Thus, the biologically active and antigenically active rPTK polypeptides that are the subject of this invention include the polypeptide represented by the entire translated nucleotide sequence of rPTK; mature rPTK; fragments thereof having a consecutive sequence of at least 5, 10, 15, 20, 25, 30, or 40 amino

30 acid residues from rPTK; amino acid sequence variants of rPTK wherein an amino acid residue has been inserted N- or C-terminal to, or within, rPTK or its fragment as defined above; amino acid sequence variants of rPTK or its fragment as defined above wherein

an amino acid residue of rPTK or its fragment as defined above has been substituted by another residue, including predetermined mutations by, *e.g.*, site-directed or PCR mutagenesis, rPTK of various animal species·such as rabbit, rat, porcine, non-human

5　primate, equine, murine, and ovine rPTK and alleles or other naturally occurring variants of the foregoing and human rPTK; derivatives of rPTK or its fragments as defined above wherein rPTK or its fragments have been covalent modified, by substitution, chemical, enzymatic, or other appropriate means, with a moiety

10　other than a naturally occurring amino acid; and glycosylation variants of rPTK (insertion of a glycosylation site or alteration of any glycosylation site by deletion, insertion, or substitution of suitable residues). Such fragments and variants exclude any polypeptide heretofore identified, including any known rPTK of any

15　animal species or any known polypeptide fragment, which is anticipatory under 35 USC §102 as well as polypeptides obvious thereover under 35 USC §103. The preferred rPTK is human mature rPTK.

An "exogenous" therapeutic compound is defined herein to mean

20　a therapeutic compound that is foreign to the mammalian patient, or homologous to a compound found in the mammalian patient but produced outside the mammalian patient.

"Extracellular domain" (ECD) of rPTK is defined herein to be any polypeptide sequence that shares a ligand binding function of

25　the ECD of the naturally occurring Rse polypeptide shown in Figure 1A or 1B; or the ECD of the naturally occurring HPTK6 polypeptide shown in Figure 2 and that may (but need not) in addition possess an antigenic function of the native extracellular domain of Rse or HPTK6. Ligand binding function of the ECD refers to the ability of

30　the polypeptide to bind at least one Rse ligand or at least one HPTK6 ligand. An antigenic function of the ECD means possession of an epitope or antigenic site that is capable of cross-reacting with antibodies raised against the polypeptide sequence of a naturally

occurring polypeptide comprising the polypeptide sequence of the ECD of Rse or HPTK6 shown in Figure 1A, 1B or Figure 2. The ECD is essentially free of the transmembrane and intracellular domains of native Rse or HPTK6, *i.e.*, has less than 1% of such domains,

5 preferably 0.5 to 0% of such domains, and more preferably 0.1 to 0% of such domains.

Ordinarily, the rPTK ECD will have an amino acid sequence having at least 75% amino acid sequence identity with the amino acid sequence of the ECD of Rse indicated in Figure 1A or 1B, or

10 the ECD of HPTK6 indicated in Figure 2, more preferably at least 80%, more preferably at least 85%, more preferably at least 90%, and most preferably at least 95%.

Thus, the ECDs of Rse or HPTK6 that are the subject of this invention include the polypeptide represented by the entire

15 translated nucleotide sequence of the ECD of Rse or HPTK6; amino acid sequence variants of the ECD of Rse or HPTK6 wherein an amino acid residue has been inserted N- or C-terminal to, or within the ECD; amino acid sequence variants of the ECD wherein an amino acid residue of the native ECD of Rse or HPTK6 has been substituted by

20 another residue, including predetermined mutations by, *e.g.*, site-directed or PCR mutagenesis, the ECD of Rse or HPTK6 of various animal species such as rabbit, rat, porcine, non-human primate, equine, murine, and ovine rPTK ECD and alleles or other naturally occurring variants of the foregoing and human ECDs; derivatives of

25 the ECD wherein the ECD has been covalently modified, by substitution, chemical, enzymatic, or other appropriate means, with a moiety other than a naturally occurring amino acid; any glycosylation variants of the ECD. Such variants exclude any polypeptide heretofore identified, which is anticipatory under 35

30 USC §102 as well as polypeptides obvious thereover under 35 USC §103. The preferred rPTK ECD is the ECD of human Rse or HPTK6.

"Ligand", when used herein, is defined to encompass any molecule, protein or non-protein, which is able to bind to the ECD

of Rse or HPTK6. The ligand may be an agonist or an antagonist to Rse or HPTK6. Generally, the ligand will activate one of the effector functions of the rPTK. For example, upon binding the ECD of the rPTK, the ligand may stimulate tyrosine kinase activity.

5 Stimulation of tyrosine kinase activity may, for example, be caused by dimerization of the rPTK which results in transphosphorylation of the kinase domain. Consequently, binding of the ligand to the receptor may result in an enhancement of cell growth and/or differentiation *in vivo* or *in vitro* or, conversely, cell growth may

10 be arrested and cell differentiation may be stimulated upon binding of the ligand to the receptor. The ligand may be the endogenous ligand for the receptor and will generally be a polypeptide. In one embodiment disclosed herein, the ligand is an antibody against the ECD of the rPTK. The preferred antibody is a humanized monoclonal

15 antibody against the ECD of rPTK. A "humanized" antibody is a chimeric antibody wherein substantially less than an intact human variable domain has been substituted by the corresponding sequence from a non-human species. Such ligands exclude any molecule heretofore identified, which is anticipatory under 35 USC §102 as

20 well as any molecule obvious thereover under 35 USC §103. The preferred ligand is the endogenous ligand to the ECD of Rse or HPTK6.

"Isolated", when used to describe the various proteins disclosed herein, means protein that has been identified and

25 separated and/or recovered from a component of its natural environment. Contaminant components of its natural environment are materials that would interfere with diagnostic or therapeutic uses for the protein, and may include enzymes, hormones, and other proteinaceous or non-proteinaceous solutes. In preferred

30 embodiments, the protein will be purified (1) to a degree sufficient to obtain at least 15 residues of N-terminal or internal amino acid sequence by use of a spinning cup sequenator, or (2) to homogeneity by SDS-PAGE under non-reducing or reducing conditions

using Coomassie blue or, preferably, silver stain. Isolated protein includes protein *in situ* within recombinant cells, since at least one component of the rPTK natural environment will not be present. Ordinarily, however, isolated protein will be prepared by

5   at least one purification step.

"Essentially pure" protein means a composition comprising at least about 90% by weight of the protein, based on total weight of the composition, preferably at least about 95% by weight. "Essentially homogeneous" protein means a composition comprising at

.0   least about 99% by weight of protein, based on total weight of the composition.

In accordance with this invention, rPTK nucleic acid or a rPTK nucleic acid molecule is RNA or DNA containing greater than ten bases that encodes a biologically active or antigenically active

.5   rPTK, is complementary to nucleic acid sequence encoding such rPTK, or hybridizes to nucleic acid sequence encoding such rPTK and remains stably bound to it under stringent conditions. The nucleic acid encoding the rPTKs, comprises nucleic acid residue nos 7-2676 of Figure 1A (*i.e.*, hRse nucleic acid); nucleic acid residue nos

!0   62-2701 of Figure 1B (*i.e.*, mRse nucleic acid); or nucleic acid residue nos 82-2820 of Figure 2 (*i.e.*, HPTK6 nucleic acid). In one embodiment, the nucleic acid sequence is selected from (a) the nucleic acid sequences of Figure 1A, 1B or Figure 2, (b) a sequence corresponding to the sequences of (a) within the scope of

25   degeneracy of the genetic code or (c) a sequence which hybridizes with a sequence defined in (a) or (b) above under stringent conditions.

Preferably, the rPTK nucleic acid molecule encodes a polypeptide sharing at least 75% sequence identity, more preferably

30   at least 80%, still more preferably at least 85%, even more preferably at least 90%, and most preferably 95%, with the rPTK amino acid sequence shown in Figure 1A, 1B or Figure 2. Preferably, the rPTK nucleic acid molecule that hybridizes to

nucl ic acid sequence encoding rPTK contains at least 20, more preferably 40, and most preferably 90 bases. Such hybridizing or complementary nucleic acid molecule, however, is further defined as being novel under 35 USC §102 and unobvious under 35 USC §103 over any prior art nucleic acid molecules.

Stringent conditions are those that (1) employ low ionic strength and high temperature for washing, for example, 0.015 M NaCl/0.0015 M sodium citrate/0.1% NaDodSO$_4$ at 50°C; (2) employ during hybridization a denaturing agent such as formamide, for example, 50% (vol/vol) formamide with 0.1% bovine serum albumin/0.1% Ficoll/0.1% polyvinylpyrrolidone/50 mM sodium phosphate buffer at pH 6.5 with 750 mM NaCl, 75 mM sodium citrate at 42°C; or (3) employ 50% formamide, 5 x SSC (0.75 M NaCl, 0.075 M sodium citrate), 50 mM sodium phosphate (pH 6.8), 0.1% sodium pyrophosphate, 5 x Denhardt's solution, sonicated salmon sperm DNA (50 µg/ml), 0.1% SDS, and 10% dextran sulfate at 42°C, with washes at 42°C in 0.2 x SSC and 0.1% SDS.

An isolated rPTK nucleic acid molecule is a nucleic acid molecule that is identified and separated from at least one contaminant nucleic acid molecule with which it is ordinarily associated in the natural source of the rPTK nucleic acid. An isolated rPTK nucleic acid molecule is other than in the form or setting in which it is found in nature. Isolated rPTK nucleic acid molecules therefore are distinguished from the rPTK nucleic acid molecule as it exists in natural cells. However, an isolated rPTK nucleic acid molecule includes rPTK nucleic acid molecules contained in cells that ordinarily express rPTK where, for example, the nucleic acid molecule is in a chromosomal location different from that of natural cells.

In accordance with this invention, rPTK ECD nucleic acid or a rPTK nucleic acid molecule encoding the ECD of rPTK is RNA or DNA containing greater than ten bases that encodes a polypeptide that shares a ligand binding function of Rse ECD or HPTK6 ECD and that

may (but need not) in addition possess an antigenic function, is complementary to nucleic acid sequence encoding such ECD, or hybridizes to nucleic acid sequence encoding such ECD and remains stably bound to it under stringent conditions. In one embodiment, the nucleic acid sequence is selected from (a) the nucleic acid sequences of SEQ ID NO: 5, SEQ ID NO: 7 or SEQ ID NO: 11, (b) a sequence corresponding to the sequences defined in (a) within the scope of degeneracy of the genetic code or (c) a sequence which hybridizes with a sequence defined in (a) or (b) above under stringent conditions.

Preferably, the rPTK ECD nucleic acid molecule encodes a polypeptide sharing at least 75% sequence identity, more preferably at least 80%, still more preferably at least 85%, even more preferably at least 90%, and most preferably 95%, with the amino acid sequences of SEQ ID NO: 5, SEQ ID NO: 7 or SEQ ID NO: 11. Such hybridizing or complementary nucleic acid molecule, however, is further defined as being novel under 35 USC §102 and unobvious under 35 USC §103 over any prior art nucleic acid molecules.

The isolated rPTK polypeptide or rPTK nucleic acid may be labeled for diagnostic and probe purposes, using a label as described and defined further below in the discussion of diagnostic assays.

The expression "control sequences" refers to DNA sequences necessary for the expression of an operably linked coding sequence in a particular host organism. The control sequences that are suitable for prokaryotes, for example, include a promoter, optionally an operator sequence, a ribosome binding site, and possibly, other as yet poorly understood sequences. Eukaryotic cells are known to utilize promoters, polyadenylation signals, and enhancers.

Nucleic acid is "operably linked" when it is placed into a functional relationship with another nucleic acid sequence. For example, DNA for a presequence or secretory leader is operably

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linked to DNA for a polypeptide if it is expressed as a preprotein that participates in the secretion of the polypeptide; a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the sequence; or a ribosome binding site is operably linked to a coding sequence if it is positioned so as to facilitate translation. Generally, "operably linked" means that the DNA sequences being linked are contiguous and, in the case of a secretory leader, contiguous and in reading phase. However, enhancers do not have to be contiguous. Linking is accomplished by ligation at convenient restriction sites. If such sites do not exist, the synthetic oligonucleotide adaptors or linkers are used in accord with conventional practice.

As used herein, the expressions "cell," "cell line," and "cell culture" are used interchangeably and all such designations include progeny. Thus, the words "transformants" and "transformed cells" include the primary subject cell and cultures derived therefrom without regard for the number of transfers. It is also understood that all progeny may not be precisely identical in DNA content, due to deliberate or inadvertent mutations. Mutant progeny that have the same function or biological activity as screened for in the originally transformed cell are included. Where distinct designations are intended, it will be clear from the context.

"Plasmids" are designated by a lower case p preceded and/or followed by capital letters and/or numbers. The starting plasmids herein are commercially available, are publicly available on an unrestricted basis, or can be constructed from such available plasmids in accord with published procedures. In addition, other equivalent plasmids are known in the art and will be apparent to the ordinary artisan.

"Restriction enzyme digestion" of DNA refers to catalytic cleavage of the DNA with an enzyme that acts only at certain locations in the DNA. Such enzymes are called restriction endonucleases, and the site for which each is specific is called a

restriction site. The various restriction enzymes used herein are commercially available and their reaction conditions, cofactors, and other requirements as established by the nzyme suppliers are used. Restriction enzymes commonly are designated by abbreviations

5   composed of a capital letter followed by other letters representing the microorganism from which each restriction enzyme originally was obtained and then a number designating the particular enzyme. In general, about 1 μg of plasmid or DNA fragment is used with about 1-2 units of enzyme in about 20 μl of buffer solution. Appropriate

10   buffers and substrate amounts for particular restriction enzymes are specified by the manufacturer. Incubation of about 1 hour at 37°C is ordinarily used, but may vary in accordance with the supplier's instructions. After incubation, protein or polypeptide is removed by extraction with phenol and chloroform, and the

15   digested nucleic acid is recovered from the aqueous fraction by precipitation with ethanol. Digestion with a restriction enzyme may be followed with bacterial alkaline phosphatase hydrolysis of the terminal 5' phosphates to prevent the two restriction-cleaved ends of a DNA fragment from "circularizing" or forming a closed

20   loop that would impede insertion of another DNA fragment at the restriction site. Unless otherwise stated, digestion of plasmids is not followed by 5' terminal dephosphorylation. Procedures and reagents for dephosphorylation are conventional as described in sections 1.56-1.61 of Sambrook _et al._, _Molecular Cloning: A_

25   _Laboratory Manual_ (New York: Cold Spring Harbor Laboratory Press, 1989).

"Recovery" or "isolation" of a given fragment of DNA from a restriction digest means separation of the digest on polyacrylamide or agarose gel by electrophoresis, identification of the fragment

30   of interest by comparison of its mobility versus that of marker DNA fragments of known molecular weight, removal of the gel section containing the desired fragment, and separation of the gel from DNA. This procedure is known generally. For example, see Lawn et

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al., Nucleic Acids Res., 9: 6103-6114 (1981), and Goeddel *et al.,* Nucleic Acids Res.8: 4057 (1980).

"Southern analysis" is a method by which the presence of DNA sequences in a restriction endonuclease digest of DNA or DNA-containing composition is confirmed by hybridization to a known, labeled oligonucleotide or DNA fragment. Southern analysis typically involves electrophoretic separation of DNA digests on agarose gels, denaturation of the DNA after electrophoretic separation, and transfer of the DNA to nitrocellulose, nylon, or another suitable membrane support for analysis with a radiolabeled, biotinylated, or enzyme-labeled probe as described in sections 9.37-9.52 of Sambrook *et al., supra.*

"Northern analysis" is a method used to identify RNA sequences that hybridize to a known probe such as an oligonucleotide, DNA fragment, cDNA or fragment thereof, or RNA fragment. The probe is labeled with a radioisotope such as ^{32}P, or by biotinylation, or with an enzyme.   The RNA to be analyzed is usually electrophoretically separated on an agarose or polyacrylamide gel, transferred to nitrocellulose, nylon, or other suitable membrane, and hybridized with the probe, using standard techniques well known in the art such as those described in sections 7.39-7.52 of Sambrook *et al., supra.*

"Ligation" refers to the process of forming phosphodiester bonds between two nucleic acid fragments. For ligation of the two fragments, the ends of the fragments must be compatible with each other. In some cases, the ends will be directly compatible after endonuclease digestion. However, it may be necessary first to convert the staggered ends commonly produced after endonuclease digestion to blunt ends to make them compatible for ligation. For blunting the ends, the DNA is treated in a suitable buffer for at least 15 minutes at 15°C with about 10 units of the Klenow fragment of DNA polymerase I or T4 DNA polymerase in the presence of the four deoxyribonucleotide triphosphates. The DNA is then purified

by phenol-chloroform extraction and ethanol precipitation. The DNA
fragments that are to be ligated together are put in solution in
about equimolar amounts. The solution will also contain ATP,
ligase buffer, and a ligase such as T4 DNA ligase at about 10 units
per 0.5 μg of DNA. If the DNA is to be ligated into a vector, the
vector is first linearized by digestion with the appropriate
restriction endonuclease(s). The linearized fragment is then
treated with bacterial alkaline phosphatase or calf intestinal
phosphatase to prevent self-ligation during the ligation step.

"Preparation" of DNA from cells means isolating the plasmid
DNA from a culture of the host cells. Commonly used methods for
DNA preparation are the large- and small-scale plasmid preparations
described in sections 1.25-1.33 of Sambrook et al., supra. After
preparation of the DNA, it can be purified by methods well known in
the art such as that described in section 1.40 of Sambrook et al.,
supra.

"Oligonucleotides" are short-length, single- or double-
stranded polydeoxynucleotides that are chemically synthesized by
known methods (such as phosphotriester, phosphite, or
phosphoramidite chemistry, using solid-phase techniques such as
described in EP 266,032 published 4 May 1988, or via
deoxynucleoside H-phosphonate intermediates as described by
Froehler et al., Nucl. Acids Res., 14: 5399-5407 (1986). Further
methods include the polymerase chain reaction defined below and
other autoprimer methods and oligonucleotide syntheses on solid
supports. All of these methods are described in Engels et al.,
Agnew. Chem. Int. Ed. Engl., 28: 716-734 (1989). These methods may
be used if the entire nucleic acid sequence of the gene is known,
or if the sequence of the nucleic acid complementary to the coding
strand is available. Alternatively, if the target amino acid
sequence is known, one may infer potential nucleic acid sequences
using known and preferred coding residues for each amino acid
residue. The oligonucleotides are then purified on polyacrylamide

gels.

The technique of "polymerase chain reaction," or "PCR," as used herein generally refers to a procedure wherein minute amounts of a specific piece of nucleic acid, RNA and/or DNA, are amplified as described in U.S. Pat. No. 4,683,195 issued 28 July 1987. Generally, sequence information from the ends of the region of interest or beyond needs to be available, such that oligonucleotide primers can be designed; these primers will be identical or similar in sequence to opposite strands of the template to be amplified. The 5' terminal nucleotides of the two primers may coincide with the ends of the amplified material. PCR can be used to amplify specific RNA sequences, specific DNA sequences from total genomic DNA, and cDNA transcribed from total cellular RNA, bacteriophage or plasmid sequences, etc. See generally Mullis et al., Cold Spring Harbor Symp. Quant. Biol., 51: 263 (1987); Erlich, ed., PCR Technology, (Stockton Press, NY, 1989). For a recent review on PCR advances, see Erlich et al., Science, 252: 1643-1650 (1991).

As used herein, PCR is considered to be one, but not the only, example of a nucleic acid polymerase reaction method for amplifying a nucleic acid test sample comprising the use of a known nucleic acid as a primer and a nucleic acid polymerase to amplify or generate a specific piece of nucleic acid.

II. Modes for Practicing the Invention

Section 1 which follows, provides methodologies for preparing full sequence rPTK, rPTK ECD, polypeptide ligands and variants thereof. The techniques disclosed in this section can be utilized for the manufacture of polypeptide ligands to the Rse and HPTK6 receptors.

1. Preparation of Natural Sequence rPTK and Variants thereof

Most of the discussion below pertains to production of rPTK by culturing cells transformed with a vector containing rPTK nucleic acid and recovering the polypeptide from the cell culture. It is further envisioned that the rPTK of this invention may be produced

by homologous recombination, as provided for in WO 91/06667 published 16 May 1991. Briefly, this method involves transforming primary mammalian cells containing endogenous rPTK gene (e.g., human cells if the desired rPTK is human) with a construct (i.e.,

5   vector) comprising an amplifiable gene [such as dihydrofolate reductase (DHFR) or others discussed below] and at least one flanking region of a length of at least about 150 bp that is homologous with a DNA sequence at the locus of the coding region of the rPTK gene to provide amplification of the rPTK gene. The

10 amplifiable gene must be at a site that does not interfere with expression of the rPTK gene. The transformation is conducted such that the construct becomes homologously integrated into the genome of the primary cells to define an amplifiable region.

Primary cells comprising the construct are then selected for

15 by means of the amplifiable gene or other marker present in the construct. The presence of the marker gene establishes the presence and integration of the construct into the host genome. No further selection of the primary cells need be made, since selection will be made in the second host. If desired, the

20 occurrence of the homologous recombination event can be determined by employing PCR and either sequencing the resulting amplified DNA sequences or determining the appropriate length of the PCR fragment when DNA from correct homologous integrants is present and expanding only those cells containing such fragments. Also if

25 desired, the selected cells may be amplified at this point by stressing the cells with the appropriate amplifying agent (such as methotrexate if the amplifiable gene is DHFR), so that multiple copies of the target gene are obtained. Preferably, however, the amplification step is not conducted until after the second

30 transformation described below.

After the selection step, DNA portions of the genome, sufficiently large to include the entire amplifiable region, are isolated from the selected primary cells. Secondary mammalian

expression host cells are then transformed with these genomic DNA portions and cloned, and clones are selected that contain the amplifiable region. The amplifiable region is then amplified by means of an amplifying agent if not already amplified in the

5 primary cells. Finally, the secondary expression host cells now comprising multiple copies of the amplifiable region containing rPTK are grown so as to express the gene and produce the protein.

A. Isolation of DNA Encoding rPTK

The DNA encoding rPTK may be obtained from any cDNA library
10 prepared from tissue believed to possess the rPTK mRNA and to express it at a detectable level. Accordingly, Rse can be conveniently obtained from a cDNA library prepared from human brain or kidney tissue and HPTK6 can be obtained from a cDNA library prepared from human adult kidney tissue. The rPTK gene may also be
15 obtained from a genomic library or by oligonucleotide synthesis as defined above assuming the complete nucleotide or amino acid sequence is known.

Libraries are screened with probes designed to identify the gene of interest or the protein encoded by it. For cDNA expression
20 libraries, suitable probes include monoclonal or polyclonal antibodies that recognize and specifically bind to the rPTK; oligonucleotides of about 20-80 bases in length that encode known or suspected portions of the rPTK cDNA from the same or different species; and/or complementary or homologous cDNAs or fragments
25 thereof that encode the same or a similar gene. Appropriate probes for screening genomic DNA libraries include, but are not limited to, oligonucleotides, cDNAs, or fragments thereof that encode the same or a similar gene, and/or homologous genomic DNAs or fragments thereof. Screening the cDNA or genomic library with the selected
30 probe may be conducted using standard procedures as described in chapters 10-12 of Sambrook et al., supra.

An alternative means to isolate the gene encoding rPTK is to use PCR methodology as described in section 14 of Sambrook et al.,

supra. This method requires the use of oligonucleotide probes that will hybridize to the rPTK. Strategies for selection of oligonucleotides are described below.

5   A preferred method of practicing this invention is to use carefully selected oligonucleotide sequences to screen cDNA libraries from various tissues, preferably mammalian brain and kidney cell lines, more preferably, human brain and human kidney cell lines.

The oligonucleotide sequences selected as probes should be of
10   sufficient length and sufficiently unambiguous that false positives are minimized. The actual nucleotide sequence(s) is usually based on conserved or highly homologous nucleotide sequences or regions of other protein tyrosine kinase molecules. The oligonucleotides may be degenerate at one or more positions. The use of degenerate
15   oligonucleotides may be of particular importance where a library is screened from a species in which preferential codon usage is not known.

The oligonucleotide must be labeled such that it can be detected upon hybridization to DNA in the library being screened.
20   The preferred method of labeling is to use ^{32}P- labeled ATP with polynucleotide kinase, as is well known in the art, to radiolabel the oligonucleotide. However, other methods may be used to label the oligonucleotide, including, but not limited to, biotinylation or enzyme labeling.

25   Of particular interest is the rPTK nucleic acid that encodes a full-length polypeptide. In some preferred embodiments, the nucleic acid sequence includes the native rPTK signal sequence. Nucleic acid having all the protein coding sequence is obtained by screening selected cDNA or genomic libraries using the deduced
30   amino acid sequence disclosed herein for the first time, and, if necessary, using conventional primer extension procedures as described in section 7.79 of Sambrook *et al.*, *supra*, to detect precursors and processing intermediates of mRNA that may not have

been reverse-transcribed into cDNA.

B. Amino Acid Sequence Variants of Native rPTK

Amino acid sequence variants of rPTK are prepared by introducing appropriate nucleotide changes into the rPTK DNA, or by synthesis of the desired rPTK polypeptide. Such variants include, for example, deletions from, or insertions or substitutions of, residues within the amino acid sequences shown for the rPTKs in Figures 1A, 1B & 2. Any combination of deletion, insertion, and substitution is made to arrive at the final construct, provided that the final construct possesses the desired characteristics. Excluded from the scope of this invention are rPTK variants or polypeptide sequences that are not novel and unobvious over the prior art. The amino acid changes also may alter post-translational processes of the rPTK, such as changing the number or position of glycosylation sites, altering the membrane anchoring characteristics, and/or altering the intracellular location of the rPTK by inserting, deleting, or otherwise affecting the leader sequence of the rPTK.

For the design of amino acid sequence variants of rPTK, the location of the mutation site and the nature of the mutation will depend on the rPTK characteristic(s) to be modified. The sites for mutation can be modified individually or in series, e.g., by (1) substituting first with conservative amino acid choices and then with more radical selections depending upon the results achieved, (2) deleting the target residue, or (3) inserting residues of the same or a different class adjacent to the located site, or combinations of options 1-3.

A useful method for identification of certain residues or regions of the rPTK polypeptide that are preferred locations for mutagenesis is called "alanine scanning mutagenesis," as described by Cunningham and Wells, Science, 244: 1081-1085 (1989). Here, a residue or group of target residues are identified (e.g., charged residues such as arg, asp, his, lys, and glu) and replaced by a

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neutral or negatively charged amino acid (most preferably alanine or polyalanine) to affect the interaction of the amino acids with the surrounding aqueous environment in or outside the cell. Those domains demonstrating functional sensitivity to the substitutions then are refined by introducing further or other variants at or for the sites of substitution. Thus, while the site for introducing an amino acid sequence variation is predetermined, the nature of the mutation *per se* need not be predetermined. For example, to optimize the performance of a mutation at a given site, ala scanning or random mutagenesis is conducted at the target codon or region and the expressed rPTK variants are screened for the optimal combination of desired activity.

There are two principal variables in the construction of amino acid sequence variants: the location of the mutation site and the nature of the mutation. These are variants of the sequences of Figures 1A, 1B & 2, and may represent naturally occurring alleles (which will not require manipulation of the rPTK DNA) or predetermined mutant forms made by mutating the DNA, either to arrive at an allele or a variant not found in nature. In general, the location and nature of the mutation chosen will depend upon the rPTK characteristic to be modified. Obviously, such variations that, for example, convert rPTK into a known receptor protein tyrosine kinase are not included within the scope of this invention, nor are any other rPTK variants or polypeptide sequences that are not novel and unobvious over the prior art.

Amino acid sequence deletions generally range from about 1 to 30 residues, more preferably about 1 to 10 residues, and typically are contiguous. Contiguous deletions ordinarily are made in even numbers of residues, but single or odd numbers of deletions are within the scope hereof. Deletions may be introduced into regions of low homology among rPTK and known rPTKs (which share the most sequence identity to the human rPTK amino acid sequence) to modify the activity of rPTK. Deletions from rPTK in areas of substantial

homology with homologous rPTK proteins will be more likely to modify the biological activity of rPTK more significantly. The number of consecutive deletions will be selected so as to preserve the tertiary structure of rPTK in the affected domain, e.g., beta-
5   pleated sheet or alpha helix.

        Amino acid sequence insertions include amino- and/or carboxyl-terminal fusions ranging in length from one residue to polypeptides containing a hundred or more residues, as well as intrasequence insertions of single or multiple amino acid residues. Intrasequence
10   insertions (i.e., insertions within the mature rPTK sequence) may range generally from about 1 to 10 residues, more preferably 1 to 5, most preferably 1 to 3. Insertions are preferably made in even numbers of residues, but this is not required. Examples of terminal insertions include mature rPTK with an N-terminal
15   methionyl residue, an artifact of the direct expression of mature rPTK in recombinant cell culture, and fusion of a heterologous N-terminal signal sequence to the N-terminus of the mature rPTK molecule to facilitate the secretion of mature rPTK from recombinant hosts. Such signal sequences generally will be
20   obtained from, and thus homologous to, the intended host cell species. Suitable sequences include STII or lpp for E. coli, alpha factor for yeast, and viral signals such as herpes gD for mammalian cells.

        Other insertional variants of the rPTK molecule include the
25   fusion to the N- or C-terminus of rPTK of immunogenic polypeptides, e.g., bacterial polypeptides such as beta-lactamase or an enzyme encoded by the E. coli trp locus, or yeast protein, and C-terminal fusions with proteins having a long half-life such as immunoglobulin constant regions (or other immunoglobulin regions),
30   albumin, or ferritin, as described in WO 89/02922 published 6 April 1989.

        A third group of variants are amino acid substitution variants. These variants have at least one amino acid residue in

the rPTK molecule removed and a different residue inserted in its place. The sites of greatest interest for substitutional mutagenesis include sites identified as the active site(s) of rPTK and sites where the amino acids found in the known analogues are substantially different in terms of side-chain bulk, charge, or hydrophobicity, but where there is also a high degree of sequence identity at the selected site within various animal rPTK species.

Other sites of interest are those in which particular residues of the rPTK obtained from various species are identical. These sites, especially those falling within a sequence of at least three other identically conserved sites, are substituted in a relatively conservative manner. Such conservative substitutions are shown in Table 1 under the heading of preferred substitutions. If such substitutions result in a change in biological activity, then more substantial changes, denominated exemplary substitutions in Table 1, or as further described below in reference to amino acid classes, are introduced and the products screened.

Table 1

Original Residue	Exemplary Substitutions	Preferred Substitutions
Ala (A)	val; leu; ile	val
Arg (R)	lys; gln; asn	lys
Asn (N)	gln; his; lys; arg	gln
Asp (D)	glu	glu
Cys (C)	ser	ser
Gln (Q)	asn	asn
Glu (E)	asp	asp
Gly (G)	pro	pro
His (H)	asn; gln; lys; arg	arg
Ile (I)	leu; val; met; ala; phe; norleucine	leu
Leu (L)	norleucine; ile; val; met; ala; phe	ile
Lys (K)	arg; gln; asn	arg

Met (M)	leu; phe; ile	leu
Phe (F)	leu; val; ile; ala	leu
Pro (P)	gly	gly
Ser (S)	thr·	thr
Thr (T)	ser	ser
Trp (W)	tyr	tyr
Tyr (Y)	trp; phe; thr; ser	phe
Val (V)	ile; leu; met; phe; ala; norleucine	leu

Substantial modifications in function or immunological identity of the rPTK are accomplished by selecting substitutions that differ significantly in their effect on maintaining (a) the structure of the polypeptide backbone in the area of the substitution, for example, as a sheet or helical conformation, (b) the charge or hydrophobicity of the molecule at the target site, or (c) the bulk of the side chain. Naturally occurring residues are divided into groups based on common side-chain properties:

(1) hydrophobic: norleucine, met, ala, val, leu, ile;

(2) neutral hydrophilic: cys, ser, thr;

(3) acidic: asp, glu;

(4) basic: asn, gln, his, lys, arg;

(5) residues that influence chain orientation: gly, pro; and

(6) aromatic: trp, tyr, phe.

Substantial modifications in enzymatic function are accomplished by deletions, or replacement of, tyrosine residues in the catalytic domain of the native rPTK as these modifications may well disrupt the tyrosine kinase activity of the receptor.

Non-conservative substitutions will entail exchanging a member of one of these classes for another. Such substituted residues also may be introduced into the conservative substitution sites or, more preferably, into the remaining (non-conserved) sites.

In one embodiment of the invention, it is desirable to inactivate one or more protease cleavage sites that are present in

the molecule. These sites are identified by inspection of the encoded amino acid sequence, in the case of trypsin, e.g., for an arginyl or lysinyl residue. When protease cleavage sites are identified, they are rendered inactive to proteolytic cleavage by

5   substituting the targeted residue with another residue, preferably a basic residue such as glutamine or a hydrophobic residue such as serine; by deleting the residue; or by inserting a prolyl residue immediately after the residue.

In another embodiment, any methionyl residues other than the

10   starting methionyl residue of the signal sequence, or any residue located within about three residues N- or C-terminal to each such methionyl residue, is substituted by another residue (preferably in accord with Table 1) or deleted. Alternatively, about 1-3 residues are inserted adjacent to such sites.

15   Any cysteine residues not involved in maintaining the proper conformation of rPTK also may be substituted, generally with serine, to improve the oxidative stability of the molecule and prevent aberrant crosslinking.

Nucleic acid molecules encoding amino acid sequence variants

20   of rPTK are prepared by a variety of methods known in the art. These methods include, but are not limited to, isolation from a natural source (in the case of naturally occurring amino acid sequence variants) or preparation by oligonucleotide-mediated (or site-directed) mutagenesis, PCR mutagenesis, and cassette

25   mutagenesis of an earlier prepared variant or a non-variant version of rPTK.

Oligonucleotide-mediated mutagenesis is a preferred method for preparing substitution, deletion, and insertion variants of rPTK DNA. This technique is well known in the art as described by

30   Adelman et al., DNA, 2: 183 (1983). Briefly, rPTK DNA is altered by hybridizing an oligonucleotide encoding the desired mutation to a DNA template, where the template is the single-stranded form of a plasmid or bacteriophage containing the unaltered or native DNA

sequence of rPTK. After hybridization, a DNA polymerase is used to synthesize an entire second complementary strand of the template that will thus incorporate the oligonucleotide primer, and will code for the selected alteration in the rPTK DNA.

5   Generally, oligonucleotides of at least 25 nucleotides in length are used. An optimal oligonucleotide will have 12 to 15 nucleotides that are completely complementary to the template on either side of the nucleotide(s) coding for the mutation. This ensures that the oligonucleotide will hybridize properly to the
10   single-stranded DNA template molecule. The oligonucleotides are readily synthesized using techniques known in the art such as that described by Crea et al., Proc. Natl. Acad. Sci. USA, 75: 5765 (1978).

The DNA template can be generated by those vectors that are
15   either derived from bacteriophage M13 vectors (the commercially available M13mp18 and M13mp19 vectors are suitable), or those vectors that contain a single-stranded phage origin of replication as described by Viera et al. Meth. Enzymol., 153: 3 (1987). Thus, the DNA that is to be mutated may be inserted into one of these
20   vectors to generate single-stranded template. Production of the single-stranded template is described in Sections 4.21-4.41 of Sambrook et al., Molecular Cloning: A Laboratory Manual (Cold Spring Harbor Laboratory Press, NY 1989).

Alternatively, single-stranded DNA template may be generated
25   by denaturing double-stranded plasmid (or other) DNA using standard techniques.

For alteration of the native DNA sequence (to generate amino acid sequence variants, for example), the oligonucleotide is hybridized to the single-stranded template under suitable
30   hybridization conditions. A DNA polymerizing enzyme, usually the Klenow fragment of DNA polymerase I, is then added to synthesize the complementary strand of the template using the oligonucleotide as a primer for synthesis. A heteroduplex molecule is thus formed

such that one strand of DNA encodes the mutated form of rPTK, and the other strand (the original template) encodes the native, unaltered sequence of rPTK. This heteroduplex molecule is then transformed into a suitable host cell, usually a prokaryote such as

5   *E. coli* JM101. After the cells are grown, they are plated onto agarose plates and screened using the oligonucleotide primer radiolabeled with ^{32}P to identify the bacterial colonies that contain the mutated DNA. The mutated region is then removed and placed in an appropriate vector for protein production, generally

.0   an expression vector of the type typically employed for transformation of an appropriate host.

The method described immediately above may be modified such that a homoduplex molecule is created wherein both strands of the plasmid contain the mutation(s). The modifications are as follows:

15   The single-stranded oligonucleotide is annealed to the single-stranded template as described above. A mixture of three deoxyribonucleotides, deoxyriboadenosine (dATP), deoxyriboguanosine (dGTP), and deoxyribothymidine (dTTP), is combined with a modified thio-deoxyribocytosine called dCTP-(aS) (which can be obtained from

20   the Amersham Corporation). This mixture is added to the template-oligonucleotide complex. Upon addition of DNA polymerase to this mixture, a strand of DNA identical to the template except for the mutated bases is generated. In addition, this new strand of DNA will contain dCTP-(aS) instead of dCTP, which serves to

25   protect it from restriction endonuclease digestion.

After the template strand of the double-stranded heteroduplex is nicked with an appropriate restriction enzyme, the template strand can be digested with *ExoIII* nuclease or another appropriate nuclease past the region that contains the site(s) to be

30   mutagenized. The reaction is then stopped to leave a molecule that is only partially single-stranded. A complete double-stranded DNA homoduplex is then formed using DNA polymerase in the presence of all four deoxyribonucleotide triphosphates, ATP, and DNA ligase.

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This homoduplex molecule can then be transformed into a suitable host cell such as *E. coli* JM101, as described above.

DNA encoding rPTK mutants with more than one amino acid to be substituted may be generated in one of several ways. If the amino acids are located close together in the polypeptide chain, they may be mutated simultaneously using one oligonucleotide that codes for all of the desired amino acid substitutions. If, however, the amino acids are located some distance from each other (separated by more than about ten amino acids), it is more difficult to generate a single oligonucleotide that encodes all of the desired changes. Instead, one of two alternative methods may be employed.

In the first method, a separate oligonucleotide is generated for each amino acid to be substituted. The oligonucleotides are then annealed to the single-stranded template DNA simultaneously, and the second strand of DNA that is synthesized from the template will encode all of the desired amino acid substitutions.

The alternative method involves two or more rounds of mutagenesis to produce the desired mutant. The first round is as described for the single mutants: wild-type DNA is used for the template, an oligonucleotide encoding the first desired amino acid substitution(s) is annealed to this template, and the heteroduplex DNA molecule is then generated. The second round of mutagenesis utilizes the mutated DNA produced in the first round of mutagenesis as the template. Thus, this template already contains one or more mutations. The oligonucleotide encoding the additional desired amino acid substitution(s) is then annealed to this template, and the resulting strand of DNA now encodes mutations from both the first and second rounds of mutagenesis. This resultant DNA can be used as a template in a third round of mutagenesis, and sc on.

PCR mutagenesis is also suitable for making amino acid variants of rPTK. While the following discussion refers to DNA, it is understood that the technique also finds application with RNA. The PCR technique generally refers to the following procedure (see

Erlich, *supra*, the chapter by R. Higuchi, p. 61-70): When small amounts of template DNA are used as starting material in a PCR, primers that differ slightly in sequence from the corresponding region in a template DNA can be used to generate relatively large quantities of a specific DNA fragment that differs from the template sequence only at the positions where the primers differ from the template. For introduction of a mutation into a plasmid DNA, one of the primers is designed to overlap the position of the mutation and to contain the mutation; the sequence of the other primer must be identical to a stretch of sequence of the opposite strand of the plasmid, but this sequence can be located anywhere along the plasmid DNA. It is preferred, however, that the sequence of the second primer is located within 200 nucleotides from that of the first, such that in the end the entire amplified region of DNA bounded by the primers can be easily sequenced. PCR amplification using a primer pair like the one just described results in a population of DNA fragments that differ at the position of the mutation specified by the primer, and possibly at other positions, as template copying is somewhat error-prone.

If the ratio of template to product material is extremely low, the vast majority of product DNA fragments incorporate the desired mutation(s). This product material is used to replace the corresponding region in the plasmid that served as PCR template using standard DNA technology. Mutations at separate positions can be introduced simultaneously by either using a mutant second primer, or performing a second PCR with different mutant primers and ligating the two resulting PCR fragments simultaneously to the vector fragment in a three (or more)-part ligation.

In a specific example of PCR mutagenesis, template plasmid DNA (1 μg) is linearized by digestion with a restriction endonuclease that has a unique recognition site in the plasmid DNA outside of the region to be amplified. Of this material, 100 ng is added to a PCR mixture containing PCR buffer, which contains the four

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deoxynucleotide triphosphates and is included in the GeneAmp® kits (obtained from Perkin-Elmer Cetus, Norwalk, CT and Emeryville, CA), and 25 pmole of each oligonucleotide primer, to a final volume of 50 µl. The reaction mixture is overlaid with 35 µl mineral oil.

5   The reaction mixture is denatured for five minutes at 100°C, placed briefly on ice, and then 1 µl *Thermus aquaticus (Taq)* DNA polymerase (5 units/µl, purchased from Perkin-Elmer Cetus) is added below the mineral oil layer. The reaction mixture is then inserted into a DNA Thermal Cycler (purchased from Perkin-Elmer Cetus)

10   programmed as follows:

     2 min. 55°C

     30 sec. 72°C, then 19 cycles of the following:

     30 sec. 94°C

     30 sec. 55°C, and

15   30 sec. 72°C.

At the end of the program, the reaction vial is removed from the thermal cycler and the aqueous phase transferred to a new vial, extracted with phenol/chloroform (50:50 vol), and ethanol precipitated, and the DNA is recovered by standard procedures.

20   This material is subsequently subjected to the appropriate treatments for insertion into a vector.

Another method for preparing variants, cassette mutagenesis, is based on the technique described by Wells *et al.*, Gene, 34: 315 (1985). The starting material is the plasmid (or other vector)

25   comprising the rPTK DNA to be mutated. The codon(s) in the rPTK DNA to be mutated are identified. There must be a unique restriction endonuclease site on each side of the identified mutation site(s). If no such restriction sites exist, they may be generated using the above-described oligonucleotide-mediated

30   mutagenesis method to introduce them at appropriate locations in the rPTK DNA. After the restriction sites have been introduced into the plasmid, the plasmid is cut at these sites to linearize it. A double-stranded oligonucleotide encoding the sequence of the

DNA between the restriction sites but containing the desired mutation(s) is synthesized using standard procedures. The two strands are synthesized separately and then hybridized together using standard techniques. This double-stranded oligonucleotide is referred to as the cassette. This cassette is designed to have 3' and 5' ends that are compatible with the ends of the linearized plasmid, such that it can be directly ligated to the plasmid. This plasmid now contains the mutated rPTK DNA sequence.

C. Insertion of Nucleic Acid into Replicable Vector

The nucleic acid (*e.g.*, cDNA or genomic DNA) encoding native or variant rPTK is inserted into a replicable vector for further cloning (amplification of the DNA) or for expression. Many vectors are available, and selection of the appropriate vector will depend on 1) whether it is to be used for DNA amplification or for DNA expression, 2) the size of the nucleic acid to be inserted into the vector, and 3) the host cell to be transformed with the vector. Each vector contains various components depending on its function (amplification of DNA or expression of DNA) and the host cell with which it is compatible. The vector components generally include, but are not limited to, one or more of the following: a signal sequence, an origin of replication, one or more marker genes, an enhancer element, a promoter, and a transcription termination sequence.

(i) Signal Sequence Component

The rPTKs of this invention may be produced recombinantly not only directly, but also as a fusion polypeptide with a heterologous polypeptide, which is preferably a signal sequence or other polypeptide having a specific cleavage site at the N-terminus of the mature protein or polypeptide. In general, the signal sequence may be a component of the vector, or it may be a part of the rPTK DNA that is inserted into the vector. The heterologous signal sequence selected should be one that is recognized and processed (*i.e.*, cleaved by a signal peptidase) by the host cell. For

prokaryotic host cells that do not recognize and process the native rPTK signal sequence, the signal sequence is substituted by a prokaryotic signal sequence selected, for exampl , from the group of the alkaline phosphatase, penicillinase, lpp, or heat-stable enterotoxin II leaders. For yeast secretion the native signal sequence may be substituted by, *e.g.*, the yeast invertase leader, alpha factor leader (including *Saccharomyces* and *Kluyveromyces* α-factor leaders, the latter described in U.S. Pat. No. 5,010,182 issued 23 April 1991), or acid phosphatase leader, the *C. albicans* glucoamylase leader (EP 362,179 published 4 April 1990), or the signal described in WO 90/13646 published 15 November 1990. In mammalian cell expression the native signal sequence (*i.e.*, the rPTK presequence that normally directs secretion of rPTK from human cells *in vivo*) is satisfactory, although other mammalian signal sequences may be suitable, such as signal sequences from other animal rPTKs, and signal sequences from secreted polypeptides of the same or related species, as well as viral secretory leaders, for example, the herpes simplex gD signal.

The DNA for such precursor region is ligated in reading frame to DNA encoding the mature rPTK.

(ii) Origin of Replication Component

Both expression and cloning vectors contain a nucleic acid sequence that enables the vector to replicate in one or more selected host cells. Generally, in cloning vectors this sequence is one that enables the vector to replicate independently of the host chromosomal DNA, and includes origins of replication or autonomously replicating sequences. Such sequences are well known for a variety of bacteria, yeast, and viruses. The origin of replication from the plasmid pBR322 is suitable for most Gram-negative bacteria, the 2μ plasmid origin is suitable for yeast, and various viral origins (SV40, polyoma, adenovirus, VSV or BPV) are useful for cloning vectors in mammalian cells. Generally, the origin of replication component is not needed for mammalian

expression vectors (the SV40 origin may typically be used only because it contains the early promoter).

Most expression vectors are "shuttle" vectors, i.e., they are capable of replication in at least one class of organisms but can
5   be transfected into another organism for expression. For example, a vector is cloned in E. coli and then the same vector is transfected into yeast or mammalian cells for expression even though it is not capable of replicating independently of the host cell chromosome.

10   DNA may also be amplified by insertion into the host genome. This is readily accomplished using Bacillus species as hosts, for example, by including in the vector a DNA sequence that is complementary to a sequence found in Bacillus genomic DNA. Transfection of Bacillus with this vector results in homologous
15   recombination with the genome and insertion of rPTK DNA. However, the recovery of genomic DNA encoding rPTK is more complex than that of an exogenously replicated vector because restriction enzyme digestion is required to excise the rPTK DNA.

(iii) Selection Gene Component
20   Expression and cloning vectors should contain a selection gene, also termed a selectable marker. This gene encodes a protein necessary for the survival or growth of transformed host cells grown in a selective culture medium. Host cells not transformed with the vector containing the selection gene will not survive in
25   the culture medium. Typical selection genes encode proteins that (a) confer resistance to antibiotics or other toxins, e.g., ampicillin, neomycin, methotrexate, or tetracycline, (b) complement auxotrophic deficiencies, or (c) supply critical nutrients not available from complex media, e.g., the gene encoding D-alanine
30   racemase for Bacilli.

One example of a selection scheme utilizes a drug to arrest growth of a host cell. Those cells that are successfully transformed with a heterologous gene express a protein conferring

drug resistance and thus survive the selection regimen. Examples of such dominant selection use the drugs neomycin (Southern et al., J. Molec. Appl. Genet., 1: 327 [1982]), mycophenolic acid (Mulligan et al., Science, 209: 1422 [1980]) or hygromycin (Sugden et al.,

5   Mol. Cell. Biol., 5: 410-413 [1985]). The three examples given above employ bacterial genes under eukaryotic control to convey resistance to the appropriate drug G418 or neomycin (geneticin), xgpt (mycophenolic acid), or hygromycin, respectively.

    Another example of suitable selectable markers for mammalian

10   cells are those that enable the identification of cells competent to take up the rPTK nucleic acid, such as DHFR or thymidine kinase. The mammalian cell transformants are placed under selection pressure that only the transformants are uniquely adapted to survive by virtue of having taken up the marker. Selection

15   pressure is imposed by culturing the transformants under conditions in which the concentration of selection agent in the medium is successively changed, thereby leading to amplification of both the selection gene and the DNA that encodes rPTK. Amplification is the process by which genes in greater demand for the production of a

20   protein critical for growth are reiterated in tandem within the chromosomes of successive generations of recombinant cells. Increased quantities of rPTK are synthesized from the amplified DNA. Other examples of amplifiable genes include metallothionein-I and -II, preferably primate metallothionein genes, adenosine

25   deaminase, ornithine decarboxylase, etc.

    For example, cells transformed with the DHFR selection gene are first identified by culturing all of the transformants in a culture medium that contains methotrexate (Mtx), a competitive antagonist of DHFR. An appropriate host cell when wild-type DHFR

30   is employed is the Chinese hamster ovary (CHO) cell line deficient in DHFR activity, prepared and propagated as described by Urlaub and Chasin, Proc. Natl. Acad. Sci. USA, 77: 4216 (1980). The transformed cells are then exposed to increased levels of

methotrexate. This leads to the synthesis of multiple copies of the DHFR gene, and, concomitantly, multiple copies of other DNA comprising the expression vectors, such as the DNA encoding rPTK. This amplification technique can be used with any otherwise

5   suitable host, e.g., ATCC No. CCL61 CHO-K1, notwithstanding the presence of endogenous DHFR if, for example, a mutant DHFR gene that is highly resistant to Mtx is employed (EP 117,060).

Alternatively, host cells [particularly wild-type hosts that contain endogenous DHFR] transformed or co-transformed with DNA

10   sequences encoding rPTK, wild-type DHFR protein, and another selectable marker such as aminoglycoside 3' phosphotransferase (APH) can be selected by cell growth in medium containing a selection agent for the selectable marker such as an aminoglycosidic antibiotic, e.g., kanamycin, neomycin, or G418.

15   See U.S. Pat. No. 4,965,199.

A suitable selection gene for use in yeast is the trp1 gene present in the yeast plasmid YRp7 (Stinchcomb et al., Nature, 282: 39 [1979]; Kingsman et al., Gene, 7: 141 [1979]; or Tschemper et al., Gene, 10: 157 [1980]). The trp1 gene provides a selection

20   marker for a mutant strain of yeast lacking the ability to grow in tryptophan, for example, ATCC No. 44076 or PEP4-1 (Jones, Genetics, 85: 12 [1977]). The presence of the trp1 lesion in the yeast host cell genome then provides an effective environment for detecting transformation by growth in the absence of tryptophan. Similarly,

25   Leu2-deficient yeast strains (ATCC 20,622 or 38,626) are complemented by known plasmids bearing the Leu2 gene.

In addition, vectors derived from the 1.6 μm circular plasmid pKD1 can be used for transformation of Kluyveromyces yeasts. Bianchi et al., Curr. Genet., 12: 185 (1987). More recently, an

30   expression system for large-scale production of recombinant calf chymosin was reported for K. lactis. Van den Berg, Bio/Technology, 8: 135 (1990). Stable multi-copy expression vectors for secretion of mature recombinant human serum albumin by industrial strains of

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Kluyveromyces have also been disclosed. Fleer *et al.,* Bio/Technology, 9: 968-975 (1991).

(iv) Promoter Component

Expression and cloning vectors usually contain a promot r that
5   is recognized by the host organism and is operably linked to the rPTK nucleic acid. Promoters are untranslated sequences located upstream (5') to the start codon of a structural gene (generally within about 100 to 1000 bp) that control the transcription and translation of particular nucleic acid sequence, such as the rPTK
10   nucleic acid sequence, to which they are operably linked. Such promoters typically fall into two classes, inducible and constitutive. Inducible promoters are promoters that initiate increased levels of transcription from DNA under their control in response to some change in culture conditions, *e.g.,* the presence
15   or absence of a nutrient or a change in temperature. At this time a large number of promoters recognized by a variety of potential host cells are well known. These promoters are operably linked to rPTK-encoding DNA by removing the promoter from the source DNA by restriction enzyme digestion and inserting the isolated promoter
20   sequence into the vector. Both the native rPTK promoter sequence and many heterologous promoters may be used to direct amplification and/or expression of the rPTK DNA. However, heterologous promoters are preferred, as they generally permit greater transcription and higher yields of rPTK as compared to the native rPTK promoter.

25   Promoters suitable for use with prokaryotic hosts include the β-lactamase and lactose promoter systems (Chang *et al.,* Nature, 275: 615 [1978]; and Goeddel *et al.,* Nature, 281: 544 [1979]), alkaline phosphatase, a tryptophan (trp) promoter system (Goeddel, Nucleic Acids Res., 8: 4057 [1980] and EP 36,776) and hybrid
30   promoters such as the tac promoter (deBoer *et al.,* Proc. Natl. Acad. Sci. USA, 80: 21-25 [1983]). However, other known bacterial promoters are suitable. Their nucleotide sequences have been published, thereby enabling a skilled worker operably to ligate

them to DNA encoding rPTK (Siebenlist et al., Cell, 20: 269 [1980])
using linkers or adaptors to supply any required restriction sites.
Promoters for use in bacterial systems also will contain a Shine-
Dalgarno (S.D.) sequence operably linked to the DNA encoding rPTK.

5   Promoter sequences are known for eukaryotes. Virtually all
eukaryotic genes have an AT-rich region located approximately 25 to
30 bases upstream from the site where transcription is initiated.
Another sequence found 70 to 80 bases upstream from the start of
transcription of many genes is a CXCAAT region where X may be any
10   nucleotide. At the 3' end of most eukaryotic genes is an AATAAA
sequence that may be the signal for addition of the poly A tail to
the 3' end of the coding sequence. All of these sequences are
suitably inserted into eukaryotic expression vectors.

Examples of suitable promoting sequences for use with yeast
15   hosts include the promoters for 3-phosphoglycerate kinase (Hitzeman
et al., J. Biol. Chem., 255: 2073 [1980]) or other glycolytic
enzymes (Hess et al., J. Adv. Enzyme Reg., 7: 149 [1968]; and
Holland, Biochemistry, 17: 4900 [1978]), such as enolase,
glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate
20   decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase,
3-phosphoglycerate mutase, pyruvate kinase, triosephosphate
isomerase, phosphoglucose isomerase, and glucokinase.

Other yeast promoters, which are inducible promoters having
the additional advantage of transcription controlled by growth
25   conditions, are the promoter regions for alcohol dehydrogenase 2,
isocytochrome C, acid phosphatase, degradative enzymes associated
with nitrogen metabolism, metallothionein, glyceraldehyde-3-phos-
phate dehydrogenase, and enzymes responsible for maltose and
galactose utilization. Suitable vectors and promoters for use in
30   yeast expression are further described in Hitzeman et al., EP
73,657A. Yeast enhancers also are advantageously used with yeast
promoters.

rPTK transcription from vectors in mammalian host cells is

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controlled, for example, by promoters obtained from the genomes of viruses such as polyoma virus, fowlpox virus (UK 2,211,504 published 5 July 1989), adenovirus (such as Adenovirus 2), bovine papilloma virus, avian sarcoma virus, cytomegalovirus, a retrovirus, hepatitis-B virus and most preferably Simian Virus 40 (SV40), from heterologous mammalian promoters, *e.g.*, the actin promoter or an immunoglobulin promoter, from heat-shock promoters, and from the promoter normally associated with the rPTK sequence, provided such promoters are compatible with the host cell systems.

The early and late promoters of the SV40 virus are conveniently obtained as an SV40 restriction fragment that also contains the SV40 viral origin of replication. Fiers *et al.*, Nature, 273:113 (1978); Mulligan and Berg, Science, 209: 1422-1427 (1980); Pavlakis *et al.*, Proc. Natl. Acad. Sci. USA, 78: 7398-7402 (1981). The immediate early promoter of the human cytomegalovirus is conveniently obtained as a HindIII E restriction fragment. Greenaway *et al.*, Gene, 18: 355-360 (1982). A system for expressing DNA in mammalian hosts using the bovine papilloma virus as a vector is disclosed in U.S. 4,419,446. A modification of this system is described in U.S. 4,601,978. See also Gray *et al.*, Nature, 295: 503-508 (1982) on expressing cDNA encoding immune interferon in monkey cells; Reyes *et al.*, Nature, 297: 598-601 (1982) on expression of human β-interferon cDNA in mouse cells under the control of a thymidine kinase promoter from herpes simplex virus; Canaani and Berg, Proc. Natl. Acad. Sci. USA, 79: 5166-5170 (1982) on expression of the human interferon $\beta1$ gene in cultured mouse and rabbit cells; and Gorman *et al.*, Proc. Natl. Acad. Sci. USA, 79: 6777-6781 (1982) on expression of bacterial CAT sequences in CV-1 monkey kidney cells, chicken embryo fibroblasts, Chinese hamster ovary cells, HeLa cells, and mouse NIH-3T3 cells using the Rous sarcoma virus long terminal repeat as a promoter.

(v) Enhancer Element Component

Transcription of a DNA encoding the rPTK of this invention by

higher eukaryotes is often increased by inserting an enhancer sequence into the vector. Enhancers are cis-acting elements of DNA, usually about from 10 to 300 bp, that act on a promoter to increase its transcription. Enhancers are relatively orientation and position independent, having been found 5' (Laimins et al., Proc. Natl. Acad. Sci. USA, 78: 993 [1981]) and 3' (Lusky et al., Mol. Cell Bio., 3: 1108 [1983]) to the transcription unit, within an intron (Banerji et al., Cell, 33: 729 [1983]), as well as within the coding sequence itself (Osborne et al., Mol. Cell Bio., 4: 1293 [1984]). Many enhancer sequences are now known from mammalian genes (globin, elastase, albumin, α-fetoprotein, and insulin). Typically, however, one will use an enhancer from a eukaryotic cell virus. Examples include the SV40 enhancer on the late side of the replication origin (bp 100-270), the cytomegalovirus early promoter enhancer, the polyoma enhancer on the late side of the replication origin, and adenovirus enhancers. See also Yaniv, Nature, 297: 17-18 (1982) on enhancing elements for activation of eukaryotic promoters. The enhancer may be spliced into the vector at a position 5' or 3' to the rPTK-encoding sequence, but is preferably located at a site 5' from the promoter.

(vi) Transcription Termination Component

Expression vectors used in eukaryotic host cells (yeast, fungi, insect, plant, animal, human, or nucleated cells from other multicellular organisms) will also contain sequences necessary for the termination of transcription and for stabilizing the mRNA. Such sequences are commonly available from the 5' and, occasionally 3', untranslated regions of eukaryotic or viral DNAs or cDNAs. These regions contain nucleotide segments transcribed as polyadenylated fragments in the untranslated portion of the mRNA encoding rPTK.

(vii) Construction and Analysis of Vectors

Construction of suitable vectors containing one or more of the above listed components employs standard ligation techniques.

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Isolated plasmids or DNA fragments are cleaved, tailored, and religated in the form desired to generate the plasmids required.

For analysis to confirm correct sequences in plasmids constructed, the ligation mixtures are used to transform *E. coli* K12 strain 294 (ATCC 31,446) and successful transformants selected by ampicillin or tetracycline resistance where appropriate. Plasmids from the transformants are prepared, analyzed by restriction endonuclease digestion, and/or sequenced by the method of Messing *et al.*, <u>Nucleic Acids Res.</u>, **9**: 309 (1981) or by the method of Maxam *et al.*, <u>Methods in Enzymology</u>, **65**: 499 (1980).

(viii) Transient Expression Vectors

Particularly useful in the practice of this invention are expression vectors that provide for the transient expression in mammalian cells of DNA encoding rPTK. In general, transient expression involves the use of an expression vector that is able to replicate efficiently in a host cell, such that the host cell accumulates many copies of the expression vector and, in turn, synthesizes high levels of a desired polypeptide encoded by the expression vector. Sambrook *et al.*, *supra*, pp. 16.17 - 16.22. Transient expression systems, comprising a suitable expression vector and a host cell, allow for the convenient positive identification of polypeptides encoded by cloned DNAs, as well as for the rapid screening of such polypeptides for desired biological or physiological properties. Thus, transient expression systems are particularly useful in the invention for purposes of identifying analogs and variants of rPTK that are biologically active rPTK.

(ix) Suitable Exemplary Vertebrate Cell Vectors

Other methods, vectors, and host cells suitable for adaptation to the synthesis of rPTK in recombinant vertebrate cell culture are described in Gething *et al.*, <u>Nature</u>, **293**: 620-625 (1981); Mantei *et al.*, <u>Nature</u>, **281**: 40-46 (1979); Levinson *et al.*; EP 117,060; and EP 117,058. A particularly useful plasmid for mammalian cell culture

expression of rPTK is pRK5 (EP pub. no. 307,247) or pSVI6B (PCT pub. no. WO 91/08291 published 13 June 1991).

D. Selection and Transformation of Host Cells

Suitable host cells for cloning or expressing the vectors
5   herein are the prokaryote, yeast, or higher eukaryote cells described above. Suitable prokaryotes for this purpose include eubacteria, such as Gram-negative or Gram-positive organisms, for example, Enterobacteriaceae such as *Escherichia*, e.g., *E. coli*, *Enterobacter*, *Erwinia*, *Klebsiella*, *Proteus*, *Salmonella*, e.g.,
10   *Salmonella typhimurium*, *Serratia*, e.g., *Serratia marcescans*, and *Shigella*, as well as *Bacilli* such as *B. subtilis* and *B. licheniformis* (e.g., *B. licheniformis* 41P disclosed in DD 266,710 published 12 April 1989), *Pseudomonas* such as *P. aeruginosa*, and *Streptomyces*. One preferred *E. coli* cloning host is *E. coli* 294
15   (ATCC 31,446), although other strains such as *E. coli* B, *E. coli* X1776 (ATCC 31,537), and *E. coli* W3110 (ATCC 27,325) are suitable. These examples are illustrative rather than limiting. Strain W3110 is a particularly preferred host or parent host because it is a common host strain for recombinant DNA product fermentations.
20   Preferably, the host cell should secrete minimal amounts of proteolytic enzymes. For example, strain W3110 may be modified to effect a genetic mutation in the genes encoding proteins, with examples of such hosts including *E. coli* W3110 strain 27C7. The complete genotype of 27C7 is *tonAΔ ptr3 phoAΔE15 Δ(argF-lac)169*
25   *ompTΔ degP41kan^r*. Strain 27C7 was deposited on 30 October 1991 in the American Type Culture Collection as ATCC No. 55,244. Alternatively, the strain of *E. coli* having mutant periplasmic protease disclosed in U.S. Pat. No. 4,946,783 issued 7 August 1990 may be employed. Alternatively, methods of cloning, e.g., PCR or
30   other nucleic acid polymerase reactions, are suitable.

In addition to prokaryotes, eukaryotic microbes such as filamentous fungi or yeast are suitable cloning or expression hosts for rPTK-encoding vectors. *Saccharomyces cerevisiae*, or common

baker's yeast, is the most commonly used among lower eukaryotic host microorganisms. However, a number of other genera, species, and strains are commonly available and useful herein, such as *Schizosaccharomyces pombe* (Beach and Nurse, Nature, 290: 140 [1981]; EP 139,383 published May 2, 1985); *Kluyveromyces* hosts (U.S. 4,943,529; Fleer *et al.*, *supra*) such as, *e.g.*, *K. lactis* [MW98-8C, CBS683, CBS4574; Louvencourt *et al.*, J. Bacteriol., 737 (1983)], *K. fragilis* (ATCC 12,424), *K. bulgaricus* (ATCC 16,045), *K. wickeramii* (ATCC 24,178), *K. waltii* (ATCC 56,500), *K. drosophilarum* (ATCC 36,906; Van den Berg *et al.*, *supra*), *K* . *thermotolerans*, and *K. marxianus*; *yarrowia* [EP 402,226]; *Pichia pastoris* (EP 183,070; Sreekrishna *et al.*, J. Basic Microbiol., 28: 265-278 [1988]); *Candida*; *Trichoderma reesia* [EP 244,234]; *Neurospora crassa* (Case *et al.*, Proc. Natl. Acad. Sci. USA, 76: 5259-5263 [1979]); *Schwanniomyces* such as *Schwanniomyces occidentalis* (EP 394,538 published 31 October 1990); and filamentous fungi such as, *e.g.*, *Neurospora*, *Penicillium*, *Tolypocladium* (WO 91/00357 published 10 January 1991), and *Aspergillus* hosts such as *A. nidulans* (Ballance *et al.*, Biochem. Biophys. Res. Commun., 112: 284-289 [1983]; Tilburn *et al.*, Gene, 26: 205-221 [1983]; Yelton *et al.*, Proc. Natl. Acad. Sci. USA, 81: 1470-1474 [1984]) and *A. niger* (Kelly and Hynes, EMBO J., 4: 475-479 [1985]).

Suitable host cells for the expression of glycosylated rPTK are derived from multicellular organisms. Such host cells are capable of complex processing and glycosylation activities. In principle, any higher eukaryotic cell culture is workable, whether from vertebrate or invertebrate culture. Examples of invertebrate cells include plant and insect cells. Numerous baculoviral strains and variants and corresponding permissive insect host cells from hosts such as *Spodoptera frugiperda* (caterpillar), *Aedes aegypti* (mosquito), *Aedes albopictus* (mosquito), *Drosophila melanogaster* (fruitfly), and *Bombyx mori* have been identified. See, *e.g.*, Luckow *et al.*, Bio/Technology, 6: 47-55 (1988); Miller *et al.*, in

Genetic Engineering, Setlow, J.K. et al., eds., Vol. 8 (Plenum Publishing, 1986), pp. 277-279; and Maeda et al., Nature, 315: 592-594 (1985). A variety of viral strains for transfection are publicly available, e.g., the L-1 variant of Autographa californica NPV and the Bm-5 strain of Bombyx mori NPV, and such viruses may be used as the virus herein according to the present invention, particularly for transfection of Spodoptera frugiperda cells.

Plant cell cultures of cotton, corn, potato, soybean, petunia, tomato, and tobacco can be utilized as hosts. Typically, plant cells are transfected by incubation with certain strains of the bacterium Agrobacterium tumefaciens, which has been previously manipulated to contain the rPTK DNA. During incubation of the plant cell culture with A. tumefaciens, the DNA encoding the rPTK is transferred to the plant cell host such that it is transfected, and will, under appropriate conditions, express the rPTK DNA. In addition, regulatory and signal sequences compatible with plant cells are available, such as the nopaline synthase promoter and polyadenylation signal sequences. Depicker et al., J. Mol. Appl. Gen., 1: 561 (1982). In addition, DNA segments isolated from the upstream region of the T-DNA 780 gene are capable of activating or increasing transcription levels of plant-expressible genes in recombinant DNA-containing plant tissue. EP 321,196 published 21 June 1989.

However, interest has been greatest in vertebrate cells, and propagation of vertebrate cells in culture (tissue culture) has become a routine procedure in recent years (Tissue Culture, Academic Press, Kruse and Patterson, editors [1973]). Examples of useful mammalian host cell lines are monkey kidney CV1 line transformed by SV40 (COS-7, ATCC CRL 1651); human embryonic kidney line (293 or 293 cells subcloned for growth in suspension culture, Graham et al., J. Gen Virol., 36: 59 [1977]); baby hamster kidney cells (BHK, ATCC CCL 10); Chinese hamster ovary cells/-DHFR (CHO, Urlaub and Chasin, Proc. Natl. Acad. Sci. USA, 77: 4216 [1980]);

mouse sertoli cells (TM4, Mather, Biol. Reprod., 23: 243-251 [1980]); monkey kidney cells (CV1 ATCC CCL 70); African green monkey kidney cells (VERO-76, ATCC CRL-1587); human cervical carcinoma cells (HELA, ATCC CCL 2); canine kidney cells (MDCK, ATCC

5 CCL 34); buffalo rat liver cells (BRL 3A, ATCC CRL 1442); human lung cells (W138, ATCC CCL 75); human liver cells (Hep G2, HB 8065); mouse mammary tumor (MMT 060562, ATCC CCL51); TRI cells (Mather et al., Annals N.Y. Acad. Sci., 383: 44-68 [1982]); MRC 5 cells; FS4 cells; and a human hepatoma line (Hep G2).

10 Host cells are transfected and preferably transformed with the above-described expression or cloning vectors of this invention and cultured in conventional nutrient media modified as appropriate for inducing promoters, selecting transformants, or amplifying the genes encoding the desired sequences.

15 Transfection refers to the taking up of an expression vector by a host cell whether or not any coding sequences are in fact expressed. Numerous methods of transfection are known to the ordinarily skilled artisan, for example, CaPO$_4$ and electroporation. Successful transfection is generally recognized when any indication

20 of the operation of this vector occurs within the host cell.

Transformation means introducing DNA into an organism so that the DNA is replicable, either as an extrachromosomal element or by chromosomal integrant. Depending on the host cell used, transformation is done using standard techniques appropriate to

25 such cells. The calcium treatment employing calcium chloride, as described in section 1.82 of Sambrook et al., supra, or electroporation is generally used for prokaryotes or other cells that contain substantial cell-wall barriers. Infection with Agrobacterium tumefaciens is used for transformation of certain

30 plant cells, as described by Shaw et al., Gene, 23: 315 (1983) and WO 89/05859 published 29 June 1989. In addition, plants may be transfected using ultrasound treatment as described in WO 91/00358 published 10 January 1991.

For mammalian cells without such cell walls, the calcium phosphate precipitation method of Graham and van der Eb, _Virology_, 52: 456-457 (1978) is preferred. General aspects of mammalian cell host system transformations have been described by Axel in U.S. 4,399,216 issued 16 August 1983. Transformations into yeast are typically carried out according to the method of Van Solingen et al., _J. Bact._, 130: 946 (1977) and Hsiao et al., _Proc. Natl. Acad. Sci. (USA)_, 76: 3829 (1979). However, other methods for introducing DNA into cells, such as by nuclear microinjection, electroporation, bacterial protoplast fusion with intact cells, or polycations, e.g., polybrene, polyornithine, etc., may also be used. For various techniques for transforming mammalian cells, see Keown et al., _Methods in Enzymology_ (1989), Keown et al., _Methods in Enzymology_, 185: 527-537 (1990), and Mansour et al., _Nature_, 336: 348-352 (1988).

E. Culturing the Host Cells

Prokaryotic cells used to produce the rPTK polypeptide of this invention are cultured in suitable media as described generally in Sambrook et al., _supra_.

The mammalian host cells used to produce the rPTK of this invention may be cultured in a variety of media. Commercially available media such as Ham's F10 (Sigma), Minimal Essential Medium ([MEM], Sigma), RPMI-1640 (Sigma), and Dulbecco's Modified Eagle's Medium ([DMEM], Sigma) are suitable for culturing the host cells. In addition, any of the media described in Ham and Wallace, _Meth. Enz._, 58: 44 (1979), Barnes and Sato, _Anal. Biochem._, 102: 255 (1980), U.S. 4,767,704; 4,657,866; 4,927,762; or 4,560,655; WO 90/03430; WO 87/00195; U.S. Pat. Re. 30,985; or U.S. 5,122,469, the disclosures of all of which are incorporated herein by reference, may be used as culture media for the host cells. Any of these media may be supplemented as necessary with hormones and/or other growth factors (such as insulin, transferrin, or epidermal growth factor), salts (such as sodium chloride, calcium, magnesium, and

phosphate), buffers (such as HEPES), nucleosides (such as adenosine and thymidine), antibiotics (such as Gentamycin™ drug), trace elements (defined as inorganic compounds usually present at final concentrations in the micromolar range), and glucose or an equivalent energy source. Any other necessary supplements may also be included at appropriate concentrations that would be known to those skilled in the art. The culture conditions, such as temperature, pH, and the like, are those previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan.

In general, principles, protocols, and practical techniques for maximizing the productivity of mammalian cell cultures can be found in Mammalian Cell Biotechnology: a Practical Approach, M. Butler, ed., IRL Press, 1991.

The host cells referred to in this disclosure encompass cells in culture as well as cells that are within a host animal.

F. Detecting Gene Amplification/Expression

Gene amplification and/or expression may be measured in a sample directly, for example, by conventional Southern blotting, Northern blotting to quantitate the transcription of mRNA (Thomas, Proc. Natl. Acad. Sci. USA, 77: 5201-5205 [1980]), dot blotting (DNA analysis), or in situ hybridization, using an appropriately labeled probe, based on the sequences provided herein. Various labels may be employed, most commonly radioisotopes, particularly ^{32}P. However, other techniques may also be employed, such as using biotin-modified nucleotides for introduction into a polynucleotide. The biotin then serves as the site for binding to avidin or antibodies, which may be labeled with a wide variety of labels, such as radionuclides, fluorescers, enzymes, or the like. Alternatively, antibodies may be employed that can recognize specific duplexes, including DNA duplexes, RNA duplexes, and DNA-RNA hybrid duplexes or DNA-protein duplexes. The antibodies in turn may be labeled and the assay may be carried out where the

duplex is bound to a surface, so that upon the formation of duplex on the surface, the presence of antibody bound to the duplex can be detected.

Gene expression, alternatively, may be measured by immunological methods, such as immunohistochemical staining of tissue sections and assay of cell culture or body fluids, to quantitate directly the expression of gene product. With immunohistochemical staining techniques, a cell sample is prepared, typically by dehydration and fixation, followed by reaction with labeled antibodies specific for the gene product coupled, where the labels are usually visually detectable, such as enzymatic labels, fluorescent labels, luminescent labels, and the like. A particularly sensitive staining technique suitable for use in the present invention is described by Hsu et al., <u>Am. J. Clin. Path.</u>, 75: 734-738 (1980).

Antibodies useful for immunohistochemical staining and/or assay of sample fluids may be either monoclonal or polyclonal, and may be prepared in any mammal. Conveniently, the antibodies may be prepared against a native rPTK polypeptide or against a synthetic peptide based on the DNA sequences provided herein as described further in Section 3 below.

G. Purification of rPTK Polypeptide

rPTK preferably is recovered from the culture medium as a secreted polypeptide, although it also may be recovered from host cell lysates when directly expressed without a secretory signal.

When rPTK is expressed in a recombinant cell other than one of human origin, the rPTK is completely free of proteins or polypeptides of human origin. However, it is necessary to purify rPTK from recombinant cell proteins or polypeptides to obtain preparations that are substantially homogeneous as to rPTK. As a first step, the culture medium or lysate is centrifuged to remove particulate cell debris. rPTK thereafter is purified from contaminant soluble proteins and polypeptides, with the following

procedures being exemplary of suitable purification procedures: by fractionation on immunoaffinity or ion-exchange columns; ethanol precipitation; reverse phase HPLC; chromatography on silica or on a cation-exchange resin·such as DEAE; chromatofocusing; SDS-PAGE; 5 ammonium sulfate precipitation; gel filtration using, for example, Sephadex G-75; and protein A Sepharose columns to, remove contaminants such as IgG.

rPTK variants in which residues have been deleted, inserted, or substituted are recovered in the same fashion as native rPTK, 10 taking account of any substantial changes in properties occasioned by the variation. For example, preparation of a rPTK fusion with another protein or polypeptide, e.g., a bacterial or viral antigen, facilitates purification; an immunoaffinity column containing antibody to the antigen can be used to adsorb the fusion 15 polypeptide. Immunoaffinity columns such as a rabbit polyclonal anti-rPTK column can be employed to absorb the rPTK variant by binding it to at least one remaining immune epitope. A protease inhibitor such as phenyl methyl sulfonyl fluoride (PMSF) also may be useful to inhibit proteolytic degradation during purification, 20 and antibiotics may be included to prevent the growth of adventitious contaminants. One skilled in the art will appreciate that purification methods suitable for native rPTK may require modification to account for changes in the character of rPTK or its variants upon expression in recombinant cell culture.

25   H. Covalent Modifications of rPTK Polypeptides

Covalent modifications of rPTK polypeptides are included within the scope of this invention. Both native rPTK and amino acid sequence variants of the rPTK may be covalently modified. One type of covalent modification included within the scope of this 30 invention is a rPTK fragment. Variant rPTK fragments having up to about 40 amino acid residues may be conveniently prepared by chemical synthesis or by enzymatic or chemical cleavage of the full-length or variant rPTK polypeptide. Other types of covalent

modifications of the rPTK or fragments thereof are introduced into the molecule by reacting targeted amino acid residues of the rPTK or fragments thereof with an organic derivatizing agent that is capable of reacting with selected side chains or the N- or C-

5   terminal residues.

Cysteinyl residues most commonly are reacted with α-haloacetates (and corresponding amines), such as chloroacetic acid or chloroacetamide, to give carboxymethyl or carboxyamidomethyl derivatives. Cysteinyl residues also are derivatized by reaction

10   with bromotrifluoroacetone, α-bromo-β-(5-imidozoyl)propionic acid, chloroacetyl phosphate, N-alkylmaleimides, 3-nitro-2-pyridyl disulfide, methyl 2-pyridyl disulfide, p-chloromercuribenzoate, 2-chloromercuri-4-nitrophenol, or chloro-7-nitrobenzo-2-oxa-1,3-diazole.

15   Histidyl residues are derivatized by reaction with diethyl-pyrocarbonate at pH 5.5-7.0 because this agent is relatively specific for the histidyl side chain. Para-bromophenacyl bromide also is useful; the reaction is preferably performed in 0.1M sodium cacodylate at pH 6.0.

20   Lysinyl and amino terminal residues are reacted with succinic or other carboxylic acid anhydrides. Derivatization with these agents has the effect of reversing the charge of the lysinyl residues. Other suitable reagents for derivatizing α-amino-containing residues include imidoesters such as methyl

25   picolinimidate; pyridoxal phosphate; pyridoxal; chloroborohydride; trinitrobenzenesulfonic acid; O-methylisourea; 2,4-pentanedione; and transaminase-catalyzed reaction with glyoxylate.

Arginyl residues are modified by reaction with one or several conventional reagents, among them phenylglyoxal, 2,3-butanedione,

30   1,2-cyclohexanedione, and ninhydrin. Derivatization of arginine residues requires that the reaction be performed in alkaline conditions because of the high pK_a of the guanidine functional group. Furthermore, these reagents may react with the groups of

lysine as well as the arginine epsilon-amino group.

The specific modification of tyrosyl residues may be made, with particular interest in introducing spectral labels into tyrosyl residues by reaction with aromatic diazonium compounds or tetranitromethane. Most commonly, N-acetylimidizole and tetranitromethane are used to form O-acetyl tyrosyl species and 3-nitro derivatives, respectively. Tyrosyl residues are iodinated using ^{125}I or ^{131}I to prepare labeled proteins for use in radioimmunoassay, the chloramine T method described above being suitable.

Carboxyl side groups (aspartyl or glutamyl) are selectively modified by reaction with carbodiimides (R-N=C=N-R'), where R and R' are different alkyl groups, such as 1-cyclohexyl-3-(2-morpholinyl-4-ethyl) carbodiimide or 1-ethyl-3-(4-azonia-4,4-dimethylpentyl) carbodiimide. Furthermore, aspartyl and glutamyl residues are converted to asparaginyl and glutaminyl residues by reaction with ammonium ions.

Derivatization with bifunctional agents is useful for crosslinking rPTK to a water-insoluble support matrix or surface for use in the method for purifying anti-rPTK antibodies, and vice-versa. Commonly used crosslinking agents include, e.g., 1,1-bis(diazoacetyl)-2-phenylethane, glutaraldehyde, N-hydroxy-succinimide esters, for example, esters with 4-azidosalicylic acid, homobifunctional imidoesters, including disuccinimidyl esters such as 3,3'-dithiobis(succinimidylpropionate), and bifunctional maleimides such as bis-N-maleimido-1,8-octane. Derivatizing agents such as methyl-3-[(p-azidophenyl)dithio]propioimidate yield photoactivatable intermediates that are capable of forming crosslinks in the presence of light. Alternatively, reactive water-insoluble matrices such as cyanogen bromide-activated carbohydrates and the reactive substrates described in U.S. 3,969,287; 3,691,016; 4,195,128; 4,247,642; 4,229,537; and 4,330,440 are employed for protein immobilization.

4,330,440 are employed for protein immobilization.

Glutaminyl and asparaginyl residues are frequently deamidated to the corresponding glutamyl and aspartyl residues, respectively. These residues are deamidated under neutral or basic conditions. The deamidated form of these residues falls within the scope of this invention.

Other modifications include hydroxylation of proline and lysine, phosphorylation of hydroxyl groups of seryl or threonyl residues, methylation of the α-amino groups of lysine, arginine, and histidine side chains (T.E. Creighton, Proteins: Structure and Molecular Properties, W.H. Freeman & Co., San Francisco, pp. 79-86 [1983]), acetylation of the N-terminal amine, and amidation of any C-terminal carboxyl group.

Another type of covalent modification of the rPTK polypeptide included within the scope of this invention comprises altering the native glycosylation pattern of the polypeptide. By altering is meant deleting one or more carbohydrate moieties found in native rPTK, and/or adding one or more glycosylation sites that are not present in the native rPTK.

Glycosylation of polypeptides is typically either N-linked or O-linked. N-linked refers to the attachment of the carbohydrate moiety to the side chain of an asparagine residue. The tripeptide sequences asparagine-X-serine and asparagine-X-threonine, where X is any amino acid except proline, are the recognition sequences for enzymatic attachment of the carbohydrate moiety to the asparagine side chain. Thus, the presence of either of these tripeptide sequences in a polypeptide creates a potential glycosylation site. O-linked glycosylation refers to the attachment of one of the sugars N-aceylgalactosamine, galactose, or xylose to a hydroxyamino acid, most commonly serine or threonine, although 5-hydroxyproline or 5-hydroxylysine may also be used.

Addition of glycosylation sites to the rPTK polypeptide is conveniently accomplished by altering the amino acid sequence such

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sequences (for N-linked glycosylation sites). The alteration may also be made by the addition of, or substitution by, one or more serine or threonine residues to the native rPTK sequence (for O-linked glycosylation sites). For ease, the rPTK amino acid sequence is preferably altered through changes at the DNA level, particularly by mutating the DNA encoding the rPTK polypeptide at preselected bases such that codons are generated that will translate into the desired amino acids. The DNA mutation(s) may be made using methods described above under the heading of "Amino Acid Sequence Variants of rPTK Polypeptide."

Another means of increasing the number of carbohydrate moieties on the rPTK polypeptide is by chemical or enzymatic coupling of glycosides to the polypeptide. These procedures are advantageous in that they do not require production of the polypeptide in a host cell that has glycosylation capabilities for N- or O-linked glycosylation. Depending on the coupling mode used, the sugar(s) may be attached to (a) arginine and histidine, (b) free carboxyl groups, (c) free sulfhydryl groups such as those of cysteine, (d) free hydroxyl groups such as those of serine, threonine, or hydroxyproline, (e) aromatic residues such as those of phenylalanine, tyrosine, or tryptophan, or (f) the amide group of glutamine. These methods are described in WO 87/05330 published 11 September 1987, and in Aplin and Wriston, CRC Crit. Rev. Biochem., pp. 259-306 (1981).

Removal of carbohydrate moieties present on the rPTK polypeptide may be accomplished chemically or enzymatically. Chemical deglycosylation requires exposure of the polypeptide to the compound trifluoromethanesulfonic acid, or an equivalent compound. This treatment results in the cleavage of most or all sugars except the linking sugar (N-acetylglucosamine or N-acetylgalactosamine), while leaving the polypeptide intact. Chemical deglycosylation is described by Hakimuddin, et al., Arch. Biochem. Biophys., 259: 52 (1987) and by Edge et al., Anal.

Biochem., 118: 131 (1981). Enzymatic cleavage of carbohydrate moieties on polypeptides can be achieved by the use of a variety of endo- and exo-glycosidases as described by Thotakura et al., Meth. Enzymol., 138: 350 (1987).

Glycosylation at potential glycosylation sites may be prevented by the use of the compound tunicamycin as described by Duskin et al., J. Biol. Chem., 257: 3105 (1982). Tunicamycin blocks the formation of protein-N-glycoside linkages.

Another type of covalent modification of rPTK comprises linking the rPTK polypeptide to one of a variety of nonproteinaceous polymers, e.g., polyethylene glycol, polypropylene glycol, or polyoxyalkylenes, in the manner set forth in U.S. Pat. Nos. 4,640,835; 4,496,689; 4,301,144; 4,670,417; 4,791,192 or 4,179,337.

rPTK also may be entrapped in microcapsules prepared, for example, by coacervation techniques or by interfacial polymerization (for example, hydroxymethylcellulose or gelatin-microcapsules and poly-[methylmethacylate] microcapsules, respectively), in colloidal drug delivery systems (for example, liposomes, albumin microspheres, microemulsions, nano-particles and nanocapsules), or in macroemulsions. Such techniques are disclosed in Remington's Pharmaceutical Sciences, 16th edition, Osol, A., Ed., (1980).

rPTK preparations are also useful in generating antibodies, as standards in assays for rPTK (e.g., by labeling rPTK for use as a standard in a radioimmunoassay, enzyme-linked immunoassay, or radioreceptor assay), in affinity purification techniques, and in competitive-type receptor binding assays when labeled with radioiodine, enzymes, fluorophores, spin labels, and the like.

Since it is often difficult to predict in advance the characteristics of a variant rPTK, it will be appreciated that some screening of the recovered variant will be needed to select the optimal variant. For example, one can screen for protein kinase

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activity using the techniques set forth in Lokker *et al.*, EMBO, **11**, 2503-2510 (1992). A change in the immunological character of the rPTK molecule, such as affinity for a given antibody, is also able to be measured by a competitive-type immunoassay. The variant is

5   assayed for changes in the suppression or enhancement of its enzymatic activity by comparison to the activity observed for native rPTK in the same assay. Other potential modifications of protein or polypeptide properties such as redox or thermal stability, hydrophobicity, susceptibility to proteolytic

10   degradation, or the tendency to aggregate with carriers or into multimers are assayed by methods well known in the art.

2.   Uses, Therapeutic Compositions and Administration of rPTK

     rPTK is believed to find therapeutic use for treating mammals via stimulation of cell growth and/or differentiation. For example,

15   Rse or HPTK6 may be used to treat neuro-degenerative diseases (e.g. senile dementia of the Alzheimer's type, peripheral neuropathies, Parkinson's disease and Huntington's disease) or diseases of the kidney (e.g., glomerulus sclerosis, which is associated with diabetes). Rse may similarly be used to generate the production of

20   platelets from megakaryocytes. Hence, the Rse may find utility for use in relation to bone marrow transplants, for example.

     The nucleic acid encoding the rPTK may be used as a diagnostic for tissue-specific typing. For example, such procedures as *in situ* hybridization, Northern and Southern blotting, and PCR

25   analysis may be used to determine whether DNA and/or RNA encoding rPTK is present in the cell type(s) being evaluated.

     Isolated rPTK polypeptide may also be used in quantitative diagnostic assays as a standard or control against which samples containing unknown quantities of rPTK may be prepared.

30   Therapeutic formulations of rPTK for treating neuro-degenerative or kidney diseases are prepared for storage by mixing rPTK having the desired degree of purity with optional physiologically acceptable carriers, excipients, or stabilizers

(<u>Remington's Pharmaceutical Sciences</u>, *supra*), in the form of lyophilized cake or aqueous solutions. Acceptable carriers, excipients or stabilizers are nontoxic to recipients at the dosages and concentrations employed, and include buffers such as phosphate, citrate, and other organic acids; antioxidants including ascorbic acid; low molecular weight (less than about 10 residues) polypeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, arginine or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrins; chelating agents such as EDTA; sugar alcohols such as mannitol or sorbitol; salt-forming counterions such as sodium; and/or nonionic surfactants such as Tween, Pluronics or polyethylene glycol (PEG).

rPTK to be used for *in vivo* administration must be sterile. This is readily accomplished by filtration through sterile filtration membranes, prior to or following lyophilization and reconstitution. rPTK ordinarily will be stored in lyophilized form or in solution.

Therapeutic rPTK compositions generally are placed into a container having a sterile access port, for example, an intravenous solution bag or vial having a stopper pierceable by a hypodermic injection needle.

The route of rPTK, or rPTK antibody administration is in accord with known methods, *e.g.*, injection or infusion by intravenous, intraperitoneal, intracerebral, intramuscular, intraocular, intraarterial, or intralesional routes, or by sustained release systems as noted below. rPTK is administered continuously by infusion or by bolus injection. rPTK antibody is administered in the same fashion, or by administration into the blood stream or lymph.

Suitable examples of sustained-release preparations include semipermeable matrices of solid hydrophobic polymers containing the

protein, which matrices are in the form of shaped articles, *e.g.*, films, or microcapsules. Examples of sustained-release matrices include polyesters, hydrogels [*e.g.*, poly(2-hydroxyethyl-methacrylate) as described by Langer *et al.*, <u>J. Biomed. Mater.</u>

5   <u>Res.</u>, **15**: 167-277 (1981) and Langer, <u>Chem. Tech.</u>, **12**: 98-105 (1982) or poly(vinylalcohol)], polylactides (U.S. 3,773,919, EP 58,481), copolymers of L-glutamic acid and gamma ethyl-L-glutamate (Sidman *et al.*, <u>Biopolymers</u>, **22**: 547-556 [1983]), non-degradable ethylene-vinyl acetate (Langer *et al.*, *supra*), degradable lactic

10 acid-glycolic acid copolymers such as the Lupron Depot™ (injectable microspheres composed of lactic acid-glycolic acid copolymer and leuprolide acetate), and poly-D-(-)-3-hydroxybutyric acid (EP 133,988).

While polymers such as ethylene-vinyl acetate and lactic acid-

15 glycolic acid enable release of molecules for over 100 days, certain hydrogels release proteins for shorter time periods. When encapsulated proteins remain in the body for a long time, they may denature or aggregate as a result of exposure to moisture at 37°C, resulting in a loss of biological activity and possible changes in

20 immunogenicity. Rational strategies can be devised for protein stabilization depending on the mechanism involved. For example, if the aggregation mechanism is discovered to be intermolecular S-S bond formation through thio-disulfide interchange, stabilization may be achieved by modifying sulfhydryl residues, lyophilizing from

25 acidic solutions, controlling moisture content, using appropriate additives, and developing specific polymer matrix compositions.

Sustained-release rPTK compositions also include liposomally entrapped rPTK. Liposomes containing rPTK are prepared by methods known *per se*: DE 3,218,121; Epstein *et al.*, <u>Proc. Natl. Acad. Sci.</u>

30 <u>USA</u>, **82**: 3688-3692 (1985); Hwang *et al.*, <u>Proc. Natl. Acad. Sci.</u> <u>USA</u>, **77**: 4030-4034 (1980); EP 52,322; EP 36,676; EP 88,046; EP 143,949; EP 142,641; Japanese patent application 83-118008; U.S. 4,485,045 and 4,544,545; and EP 102,324. Ordinarily the liposomes

are of the small (about 200-800 Angstroms) unilamellar type in which the lipid content is greater than about 30 mol. % cholesterol, the selected proportion being adjusted for the optimal rPTK therapy.

5   An effective amount of rPTK to be employed therapeutically will depend, for example, upon the therapeutic objectives, the route of administration, and the condition of the patient. Accordingly, it will be necessary for the therapist to titer the dosage and modify the route of administration as required to obtain

10   the optimal therapeutic effect. A typical daily dosage might range from about 1 µg/kg to up to 100 mg/kg or more, depending on the factors mentioned above. Typically, the clinician will administer rPTK until a dosage is reached that achieves the desired effect. The progress of this therapy is easily monitored by conventional

15   assays.

3.   rPTK Antibody Preparation

The antibodies of this invention are obtained by routine screening. Polyclonal antibodies to the rPTK generally are raised in animals by multiple subcutaneous (sc) or intraperitoneal (ip)

20   injections of the rPTK and an adjuvant. It may be useful to conjugate the rPTK or a fragment containing the target amino acid sequence to a protein that is immunogenic in the species to be immunized, e.g., keyhole limpet hemocyanin, serum albumin, bovine thyroglobulin, or soybean trypsin inhibitor using a bifunctional or

25   derivatizing agent, for example, maleimidobenzoyl sulfosuccinimide ester (conjugation through cysteine residues), N-hydroxysuccinimide (through lysine residues), glutaraldehyde, succinic anhydride, $SOCl_2$, or $R^1N = C = NR$, where R and R^1 are different alkyl groups.

The route and schedule of the host animal or cultured

30   antibody-producing cells therefrom are generally in keeping with established and conventional techniques for antibody stimulation and production. While mice are frequently employed as the test model, it is contemplated that any mammalian subject including

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human subjects or antibody-producing cells obtained therefrom can be manipulated according to the processes of this invention to serve as the basis for production of mammalian, including human, hybrid cell lines.

Animals are typically immunized against the immunogenic conjugates or derivatives by combining 1 mg or 1 μg of conjugate (for rabbits or mice, respectively) with 3 volumes of Freund's complete adjuvant and injecting the solution intradermally at multiple sites. One month later the animals are boosted with 1/5 to 1/10 the original amount of conjugate in Freund's complete adjuvant (or other suitable adjuvant) by subcutaneous injection at multiple sites. 7 to 14 days later animals are bled and the serum is assayed for anti-rPTK titer. Animals are boosted until the titer plateaus. Preferably, the animal is boosted with the conjugate of the same rPTK, but conjugated to a different protein and/or through a different cross-linking agent. Conjugates also can be made in recombinant cell culture as protein fusions. Also, aggregating agents such as alum are used to enhance the immune response.

After immunization, monoclonal antibodies are prepared by recovering immune cells (typically spleen cells or lymphocytes from lymph node tissue) from immunized animals and immortalizing the cells in conventional fashion, e.g., by fusion with myeloma cells or by Epstein-Barr (EB)-virus transformation and screening for clones expressing the desired antibody. The hybridoma technique described originally by Kohler and Milstein, Eur. J. Immunol., 6: 511 (1976), and also described by Hammerling et al., In: Monoclonal Antibodies and T-Cell Hybridomas, Elsevier, N.Y., pp. 563-681 (1981) has been widely applied to produce hybrid cell lines that secrete high levels of monoclonal antibodies against many specific antigens.

It is possible to fuse cells of one species with another. However, it is preferable that the source of the immunized antibody

producing cells and the myeloma be from the same species.

The hybrid cell lines can be maintained in culture in cell culture media. The cell lines of this invention can be selected and/or maintained in a composition comprising the continuous cell line in hypoxanthine-aminopterin-thymidine (HAT) medium. In fact, once the hybridoma cell line is established, it can be maintained on a variety of nutritionally adequate media. Moreover, the hybrid cell lines can be stored and preserved in any number of conventional ways, including freezing and storage under liquid nitrogen. Frozen cell lines can be revived and cultured indefinitely with resumed synthesis and secretion of monoclonal antibody.

The secreted antibody is recovered from tissue culture supernatant by conventional methods such as precipitation, ion exchange chromatography, affinity chromatography, or the like. The antibodies described herein are also recovered from hybridoma cell cultures by conventional methods for purification of IgG or IgM, as the case may be, that heretofore have been used to purify these immunoglobulins from pooled plasma, e.g., ethanol or polyethylene glycol precipitation procedures. The purified antibodies are sterile filtered, and optionally are conjugated to a detectable marker such as an enzyme or spin label for use in diagnostic assays of the rPTK in test samples.

While routinely mouse monoclonal antibodies are used, the invention is not so limited; in fact, human antibodies may be used and may prove to be preferable. Such antibodies can be obtained by using human hybridomas (Cote et al., Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, p. 77 [1985]). In fact, according to the invention, techniques developed for the production of chimeric antibodies (Morrison et al., Proc. Natl. Acad. Sci., 81: 6851 [1984]); Neuberger et al., Nature, 312: 604 [1984]; Takeda et al., Nature, 314: 452 [1985]; EP 184,187; EP 171,496; EP 173,494; PCT WO 86/01533; Shaw et al., J. Nat. Canc. Inst., 80: 1553-1559 [1988];

Morrison, _Science_, 229: 1202-1207 [1985]; and Oi _et al._, _BioTechniques_, 4: 214 [1986]) by splicing the genes from a mouse antibody molecule of appropriate antigen specificity together with genes from a human antibody molecule of appropriate biological

5   activity (such as ability to activate human complement and mediate ADCC) can be used; such antibodies are within the scope of this invention.

In a preferred embodiment of the invention, humanized antibodies are used to reduce or eliminate any anti-globulin immune

10 response in humans. As used herein, the term "humanized" antibody is an embodiment of chimeric antibodies wherein substantially less than an intact human variable domain has been substituted by the corresponding sequence from a non-human species. In practice, humanized antibodies are typically human antibodies in which some

15 amino acid residues from the complementarity determining regions (CDRs), the hypervariable regions in the variable domains which are directly involved with formation of the antigen-binding site, and possibly some amino acids from the framework regions (FRs), the regions of sequence that are somewhat conserved within the variable

20 domains, are substituted by residues from analogous sites in rodent antibodies. The construction of humanized antibodies is described in Riechmann _et al._, _Nature_, 332: 323-327 (1988), Queen _et al._, _Proc. Natl. Acad. Sci. USA_, 86: 10029-10033 (1989), Co _et al._, _Proc. Natl. Acad. Sci. USA_, 88: 2869-2873 (1991), Gorman _et al._,

25 _Proc. Natl. Acad. Sci._, 88: 4181-4185 (1991), Daugherty _et al._, _Nucleic Acids Res._, 19: 2471-2476 (1991), Brown _et al._, _Proc. Natl. Acad. Sci. USA_, 88: 2663-2667 (1991), Junghans _et al._, _Cancer Res._, 50: 1495-1502 (1990), Fendly _et al._, _Cancer Res._, 50: 1550-1558 (1990) and in PCT application WO 89/06692.

30 In some cases, substituting CDRs from rodent antibodies for the human CDRs in human frameworks is sufficient to transfer high antigen binding affinity (Jones _et al._, _Nature_, 321: 522-525 [1986]; Verhoeyen _et al._, _Science_, 239: 1534-1536 [1988]) whereas

in other cases it is necessary to additionally replace one (Riechmann *et al., supra*) or several (Queen *et al., supra*) FR residues. See also Co *et al., supra*.

In a particularly preferred embodiment of the invention, the humanized antibodies are designed and constructed according to the methods described in PCT application WO 92/22653, the entire disclosure of which is specifically incorporated herein by reference.

Techniques for creating recombinant DNA versions of the antigen-binding regions of antibody molecules (known as Fab fragments), which bypass the generation of monoclonal antibodies, are encompassed within the practice of this invention. One extracts antibody-specific messenger RNA molecules from immune system cells taken from an immunized animal, transcribes these into complementary DNA (cDNA), and clones the cDNA into a bacterial expression system. One example of such a technique suitable for the practice of this invention was developed by researchers at Scripps/Stratagene, and incorporates a proprietary bacteriophage lambda vector system that contains a leader sequence that causes the expressed Fab protein to migrate to the periplasmic space (between the bacterial cell membrane and the cell wall) or to be secreted. One can rapidly generate and screen great numbers of functional Fab fragments for those that bind the antigen. Such rPTK-binding molecules (Fab fragments with specificity for the rPTK) are specifically encompassed within the term "antibody" as it is defined, discussed, and claimed herein.

The antibody preferably does not cross-react with other known receptor protein tyrosine kinases.

4. Uses of rPTK Antibodies

rPTK antibodies may be used as ligands to the rPTK and are also useful in diagnostic assays for rPTK, *e.g.*, detecting its expression in specific cells, tissues, or serum. The antibodies are labeled in the same fashion as rPTK described above and/or are

immobilized on an insoluble matrix. In one embodiment of a receptor binding assay, an antibody composition that binds to all or a selected plurality of members of the rPTK family is immobilized on an insoluble matrix, the test sample is contacted with the immobilized antibody composition to adsorb all rPTK family members, and then the immobilized family members are contacted with a plurality of antibodies specific for each member, each of the antibodies being individually identifiable as specific for a predetermined family member, as by unique labels such as discrete fluorophores or the like. By determining the presence and/or amount of each unique label, the relative proportion and amount of each family member can be determined.

The antibodies of this invention are also useful in passively immunizing patients.

rPTK antibodies also are useful for the affinity purification of rPTK or rPTK ECD from recombinant cell culture or natural sources. rPTK antibodies that do not detectably cross-react with other receptor protein tyrosine kinases can be used to purify rPTK or rPTK ECD free from these other known proteins.

Suitable diagnostic assays for rPTK and its antibodies are well known *per se*. For example, competitive, sandwich and steric inhibition immunoassay techniques are useful. The competitive and sandwich methods employ a phase-separation step as an integral part of the method while steric inhibition assays are conducted in a single reaction mixture. Fundamentally, the same procedures are used for the assay of rPTK and for substances that bind rPTK, although certain methods will be favored depending upon the molecular weight of the substance being assayed. Therefore, the substance to be tested is referred to herein as an analyte, irrespective of its status otherwise as an antigen or antibody, and proteins that bind to the analyte are denominated binding partners, whether they be antibodies, cell surface receptors, or antigens.

Analytical methods for rPTK or its antibodies all use one or

more of the following reagents: labeled analyte analogue, immobilized analyte analogue, labeled binding partner, immobilized binding partner, and steric conjugates. The labeled reagents also are known as "tracers."

5   The label used (and this is also useful to label rPTK nucleic acid for use as a probe) is any detectable functionality that does not interfere with the binding of analyte and its binding partner. Numerous labels are known for use in immunoassay, examples including moieties that may be detected directly, such as

10   fluorochrome, chemiluminscent, and radioactive labels, as well as moieties, such as enzymes, that must be reacted or derivatized to be detected. Examples of such labels include the radioisotopes ^{32}P, ^{14}C, ^{125}I, ^{3}H, and ^{131}I, fluorophores such as rare earth chelates or fluorescein and its derivatives, rhodamine and its derivatives,

15   dansyl, umbelliferone, luciferases, e.g., firefly luciferase and bacterial luciferase (U.S. Pat. No. 4,737,456), luciferin, 2,3-dihydrophthalazinediones, malate dehydrogenase, urease, peroxidase such as horseradish peroxidase (HRP), alkaline phosphatase, β-galactosidase, glucoamylase, lysozyme, saccharide oxidases, e.g.,

20   glucose oxidase, galactose oxidase, and glucose-6-phosphate dehydrogenase, heterocyclic oxidases such as uricase and xanthine oxidase, coupled with an enzyme that employs hydrogen peroxide to oxidize a dye precursor such as HRP, lactoperoxidase, or microperoxidase, biotin/avidin, spin labels, bacteriophage labels,

25   stable free radicals, and the like.

Those of ordinary skill in the art will know of other suitable labels that may be employed in accordance with the present invention. The binding of these labels to rPTK, antibodies, or fragments thereof can be accomplished using standard techniques

30   commonly known to those of ordinary skill in the art. For instance, coupling agents such as dialdehydes, carbodiimides, dimaleimides, bis-imidates, bis-diazotized benzidine, and the like may be used to tag the polypeptide with the above-described

fluorescent, chemiluminescent, and enzyme labels. See, for example, U.S. Pat. Nos. 3,940,475 (fluorimetry) and 3,645,090 (enzymes); Hunter et al., Nature, 144: 945 (1962); David et al., Biochemistry, 13: 1014-1021 (1974); Pain et al., J. Immunol. Methods, 40: 219-230 (1981); Nygren, J. Histochem. and Cytochem., 30: 407-412 (1982); O'Sullivan et al., Methods in Enzymology, ed. J.J. Langone and H. Van Vunakis, Vol. 73 (Academic Press, New York, New York, 1981), pp. 147-166; Kennedy et al., Clin. Chim. Acta, 70: 1-31 (1976); and Schurs et al., Clin. Chim. Acta, 81: 1-40 (1977). Coupling techniques mentioned in the lattermost reference are the glutaraldehyde method, the periodate method, the dimaleimide method, and the m-maleimidobenzyl-N-hydroxysuccinimide ester method.

In the practice of the present invention, enzyme labels are a preferred embodiment. No single enzyme is ideal for use as a label in every conceivable assay. Instead, one must determine which enzyme is suitable for a particular assay system. Criteria important for the choice of enzymes are turnover number of the pure enzyme (the number of substrate molecules converted to product per enzyme site per unit of time), purity of the enzyme preparation, sensitivity of detection of its product, ease and speed of detection of the enzyme reaction, absence of interfering factors or of enzyme-like activity in the test fluid, stability of the enzyme and its conjugate, availability and cost of the enzyme and its conjugate, and the like. Included among the enzymes used as preferred labels in the assays of the present invention are alkaline phosphatase, HRP, beta-galactosidase, urease, glucose oxidase, glucoamylase, malate dehydrogenase, and glucose-6-phosphate dehydrogenase. Urease is among the more preferred enzyme labels, particularly because of chromogenic pH indicators that make its activity readily visible to the naked eye.

Immobilization of reagents is required for certain assay methods. Immobilization entails separating the binding partner

from any analyte that remains free in solution. This conventionally is accomplished by either insolubilizing the binding partner or analyte analogue before the assay procedure, as by adsorption to a water-insoluble matrix or surface (Bennich et al.,

5   U.S. 3,720,760), by covalent coupling (for example, using glutaraldehyde cross-linking), or by insolubilizing the partner or analogue afterward, e.g., by immunoprecipitation.

Other assay methods, known as competitive or sandwich assays, are well established and widely used in the commercial diagnostics

10   industry.

Competitive assays rely on the ability of a tracer analogue to compete with the test sample analyte for a limited number of binding sites on a common binding partner. The binding partner generally is insolubilized before or after the competition and then

15   the tracer and analyte bound to the binding partner are separated from the unbound tracer and analyte. This separation is accomplished by decanting (where the binding partner was preinsolubilized) or by centrifuging (where the binding partner was precipitated after the competitive reaction). The amount of test

20   sample analyte is inversely proportional to the amount of bound tracer as measured by the amount of marker substance. Dose-response curves with known amounts of analyte are prepared and compared with the test results to quantitatively determine the amount of analyte present in the test sample. These assays are

25   called ELISA systems when enzymes are used as the detectable markers.

Another species of competitive assay, called a "homogeneous" assay, does not require a phase separation. Here, a conjugate of an enzyme with the analyte is prepared and used such that when

30   anti-analyte binds to the analyte the presence of the anti-analyte modifies the enzyme activity. In this case, rPTK or its immunologically active fragments are conjugated with a bifunctional organic bridge to an enzyme such as peroxidase. Conjugates are

selected for use with anti-rPTK so that binding of the anti-rPTK inhibits or potentiates the enzyme activity of the label. This method *per se* is widely practiced under the name of EMIT.

Steric conjugates are used in steric hindrance methods for homogeneous assay. These conjugates are synthesized by covalently linking a low-molecular-weight hapten to a small analyte so that antibody to hapten substantially is unable to bind the conjugate at the same time as anti-analyte. Under this assay procedure the analyte present in the test sample will bind anti-analyte, thereby allowing anti-hapten to bind the conjugate, resulting in a change in the character of the conjugate hapten, *e.g.*, a change in fluorescence when the hapten is a fluorophore.

Sandwich assays particularly are useful for the determination of rPTK or rPTK antibodies. In sequential sandwich assays an immobilized binding partner is used to adsorb test sample analyte, the test sample is removed as by washing, the bound analyte is used to adsorb labeled binding partner, and bound material is then separated from residual tracer. The amount of bound tracer is directly proportional to test sample analyte. In "simultaneous" sandwich assays the test sample is not separated before adding the labeled binding partner. A sequential sandwich assay using an anti-rPTK monoclonal antibody as one antibody and a polyclonal anti-rPTK antibody as the other is useful in testing samples for rPTK activity.

The foregoing are merely exemplary diagnostic assays for rPTK and antibodies. Other methods now or hereafter developed for the determination of these analytes are included within the scope hereof, including the bioassays described above.

5.   rPTK Ligand Preparation

As discussed above, rPTK ligands can comprise antibodies (including polyclonal antibodies, monoclonal antibodies and humanized monoclonal antibodies) against the rPTK. Other protein and non-protein ligands are also contemplated within the scope of

the invention.

The ligand preferably constitutes the endogenous ligand to the rPTK. In order to isolate the endogenous rPTK ligand, primary cells purified from natural sources (*e.g.*, blood tissue extracts or urine) or cell lines expressing the ligands are screened for the ligand. Cells used to isolate the ligands may, for example, be selected from human kidney and brain cells. Cell lines can be established using well known techniques such as immortalization of the cells via transformation with viral DNA (*e.g.*, SV40 DNA).

The endogenous ligand can then be identified and isolated using techniques which have been established in the art. For example, the techniques disclosed in WO/92/20798 can be used to isolate the ligand to the rPTK. Generally, the ligand will be recovered from a cellular membrane fraction or a secreted form of the ligand will be isolated from the culture medium. Accordingly, the culture medium or lysate is centrifuged to remove particulate cell debris. The ligand is then purified from the soluble protein fraction or the membrane fraction of the culture lysate by biochemical separation. The following procedures are exemplary of suitable purification procedures: fractionation on an immunoaffinity or ion-exchange column; ethanol precipitation; reversed phase HPLC; chromatography on silica, Heparin Sepharose or on a cation exchange resin such as DEAE; chromatofocusing; SDS-PAGE; ammonium sulfate precipitation; and gel filtration using, for example, Sephadex G-75. Each of the fractions can then be assayed for its ability to phosphorylate the rPTK (see Example 1 for a suitable assay for tyrosine kinase activity), in order to isolate the fraction containing the ligand to the rPTK. Further purification of the fraction can then be carried out as required.

Alternatively, the techniques used by Flanagan *et al.*, Cell, 63: 185-194 (1990) can be carried out. Flanagan *et al.* isolated the ligand to the c-kit proto-oncogene by genetically fusing the c-kit ECD to placental alkaline phosphatase thereby forming a soluble

receptor affinity reagent with an enzyme tag that could be readily traced. Binding of the fusion proteins is detectable by the enzymatic activity of the alkaline phosphatase secreted into the medium. The fusion protein so formed, termed APtag-KIT, binds with high affinity to cell lines expressing the ligand of interest. The bound cells are then isolated from the APtag-KIT complex. Accordingly, a chimeric nucleic acid construct encoding the ECD of Rse or the ECD of HPTK6 fused to the secretable alkaline phosphatase marker can be generated.

To clone the cDNA that encodes the ligand, a cDNA library is constructed from the isolated cells in a suitable expression vector, such as the vectors discussed earlier herein. The library is then transfected into host cells (see above) and cells having the ligand on their surface are detected using the techniques of Flanagan *et al.* Single cell suspensions are incubated with the APtag-KIT and, after removing APtag-KIT proteins which are not bound to the cells by centrifugation, cells are panned on plates coated with antibodies against alkaline phosphatase (Seed *et al.*, Proc. Natl. Acad. Sci., 84:, 3365-69 [1987]). Cells to which the antibodies are bound are isolated and the DNA is extracted therefrom using techniques available to the skilled artisan.

6. Uses, Therapeutic Compositions and Administration of rPTK Ligand

rPTK ligands are believed to find therapeutic use for treating mammals via stimulation of cell growth and/or differentiation. For example, Rse ligand may be used to treat neuro-degenerative diseases (*e.g.* senile dementia of the Alzheimer's type, peripheral neuropathies, Parkinson's disease and Huntington's disease) or diseases of the kidney (*e.g.*, glomerulus sclerosis, which is associated with diabetes). Rse ligand may also be used to generate the production of platelets from megakaryocytes. Like Rse ligand, HPTK6 ligand may be used to treat kidney diseases, such as glomerulus sclerosis. An antagonist ligand for HPTK6 may find

therapeutic use in the treatment of cancer, *e.g.* breast cancer.

Therapeutic formulations of rPTK ligand are prepared for storage by mixing the ligand having the desired degree of purity with optional physiologically acceptable carriers, excipients, or

5   stabilizers (<u>Remington's Pharmaceutical Sciences</u>, *supra*), in the form of lyophilized cake or aqueous solutions.

rPTK ligand to be used for *in vivo* administration must be sterile.   This is readily accomplished by filtration through sterile filtration membranes, prior to or following lyophilization

10   and reconstitution.   rPTK ligand ordinarily will be stored in lyophilized form or in solution.

Therapeutic rPTK ligand compositions generally are placed into a container having a sterile access port, for example, an intravenous solution bag or vial having a stopper pierceable by a

15   hypodermic injection needle.

The route of rPTK ligand administration is in accord with known methods, *e.g.*, injection or infusion by intravenous, intraperitoneal, intracerebral, intramuscular, intraocular, intraarterial, or intralesional routes, or by sustained release

20   systems as noted below.   rPTK ligand is administered continuously by infusion or by bolus injection.

An effective amount of rPTK ligand to be employed therapeutically will depend, for example, upon the therapeutic objectives, the route of administration, and the condition of the

25   patient.   Accordingly, it will be necessary for the therapist to titer the dosage and modify the route of administration as required to obtain the optimal therapeutic effect.   A typical daily dosage might range from about 1 μg/kg to up to 100 mg/kg or more, depending on the factors mentioned above. Typically, the clinician

30   will administer rPTK ligand until a dosage is reached that achieves the desired effect.   The progress of this therapy is easily monitored by conventional assays.

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7.   <u>Uses, Therapeutic Compositions and Administration of rPTK ECD</u>

As discussed above, rPTK ECD can be used for the identification and isolation of ligands to the rPTKs using the techniques disclosed in Flanagan *et al.*, *supra*, for example.

rPTK ECD is also believed to find use as a therapeutic compound for removal of excess systemic or tissue-localized rPTK ligand which has been administered to a patient. Removal of excess ligand is particularly desirably where the ligand may be toxic to the patient. The rPTK ECD acts to bind the ligand in competition with endogenous rPTKs in the patient. Similarly, it is contemplated that the rPTK ECD can be administered to a patient simultaneously, or subsequent to, administration of the ligand in the form of a sustained release composition. The ECD acts as a soluble binding protein for the ligand, thereby extending the half-life of the ligand. Also, the ECD may constitute a ligand to the receptor in so far as it is able to bind to, and activate, the ECD of an adjacent membrane bound rPTK. Accordingly, the ECD may be used as a ligand to the rPTK.

The nucleic acid encoding the rPTK ECD may be used as a diagnostic for tissue-specific typing.   For example, such procedures as *in situ* hybridization, Northern and Southern blotting, and PCR analysis may be used to determine whether DNA and/or RNA encoding rPTK is present in the cell type(s) being evaluated.

Therapeutic formulations of rPTK ECD are prepared for storage by mixing rPTK ECD having the desired degree of purity with optional physiologically acceptable carriers, excipients, or stabilizers (<u>Remington's Pharmaceutical Sciences</u>, *supra*), in the form of lyophilized cake or aqueous solutions.

rPTK ECD to be used for *in vivo* administration must be sterile.   This is readily accomplished by filtration through sterile filtration membranes, prior to or following lyophilization and reconstitution.   rPTK ECD ordinarily will be stored in

lyophilized form or in solution.

    Therapeutic rPTK ECD compositions generally are placed into a container having a sterile access port, for example, an intravenous solution bag or vial having a stopper pierceable by a hypodermic injection needle.

    The route of rPTK ECD administration is in accord with known methods, *e.g.*, injection or infusion by intravenous, intraperitoneal, intracerebral, intramuscular, intraocular, intraarterial, or intralesional routes, or by sustained release systems as noted below. rPTK ECD is administered continuously by infusion or by bolus injection.

    An effective amount of rPTK ECD to be employed therapeutically will depend, for example, upon the therapeutic objectives, the route of administration, and the condition of the patient. Accordingly, it will be necessary for the therapist to titer the dosage and modify the route of administration as required to obtain the optimal therapeutic effect. A typical daily dosage might range from about 1 μg/kg to up to 100 mg/kg or more, depending on the factors mentioned above. Typically, the clinician will administer rPTK ECD until a dosage is reached that achieves the desired effect. The progress of this therapy is easily monitored by conventional assays.

    The following examples are offered by way of illustration and not by way of limitation. The disclosures of all literature references cited in the specification are expressly incorporated herein by reference.

<div align="center">

EXAMPLE 1

Isolation and Characterization of Rse

</div>

A.   cDNA Cloning and Sequencing

    Degenerate oligodeoxyribonucleotide primers were designed to

sequences encoding conserved amino acids in tyrosine kinases (Lai
et al., supra). These primers were used to amplify fragments of
tyrosine kinase containing genes from cDNA prepared from human
brain RNA. Amplified fragments were cloned and sequenced. Nested

5   oligodeoxyribonucleotide primers (pair A: 5'-
CGGATCCAC(AC)G(ATGC)GA(CT)(CT)T (SEQ ID NO: 13) and 5'-
GGAATTCC(TC)TC(AT)GGAG(CT)(AG)TCCA(TC)(TC)T (SEQ ID NO: 14); pair
B: 5'-CGGATCCATCCACAGAGATGT (SEQ ID NO: 15) and 5'-
GGAATTCCAAAGGACCA(GC)AC(GA)TC) (SEQ ID NO: 16) were used to amplify

10 fragments of cDNA prepared from human brain RNA. Amplified DNA
fragments were cloned as BamHI and EcoRI inserts in pUC19 (see
Hanks et al., supra). Amplification reactions were performed using
Taq DNA polymerase in a Perkin-Elmer 480 thermocycler, 40 cycles of
94°C for 30 seconds, 45°C for 30 seconds, and 72°C for 1 minute;

15 primer-pair B was added following cycle 20. Recombinants were
identified and sequenced using the dideoxynucleotide method. A 50
base single-stranded oligodeoxyribonucleotide probe
(5'-GACCGTGTGTGTGGCTGACTTTGGACTCTCCTGGAAGATC (SEQ ID NO: 17)) was
used as a probe to screen 1.2×10^6 plaques from a random-primed

20 lambda gt10 library prepared from RNA isolated from human fetal
brain. Conditions for plating libraries, hybridizing and washing
filters were as previously described (Godowski, et al., Proc. Natl.
Acad. Sci. 86: 8083-8087 [1989]). One positive plaque was obtained,
with an insert size of approximately 1.2 Kb. An

25 oligodeoxyribonucleotide probe (5'-
GGCTGTGCCTCCAAATTGCCCGTCAAGTGGCTGGCCCTGG (SEQ ID NO: 18)) based on
sequence obtained from the 5' end of the 1.2 Kb clone was used to
screen 1.2×10^6 plaques from an oligo dT-primed lambda gt10
library prepared from RNA from the Hep 3B cell line. The inserts

30 from 15 positive plaques were characterized, and the largest
insert, approximately 3.5 Kb in length, was sequenced. An
oligodeoxyribonucleotide primer
(5'-AGCCGGTGAAGCTGAACTGCAGTGTGGAGGGGATGGAGGAGCCTGACATC (SEQ ID NO:

19)) based on sequence from the 5' region of the 3.5 Kb clone was used to screen 1.2 x 10⁶ plaques from a second lambda gt10 Hep 3B library. Four clones were obtained, and one of these contained a 3.0 Kb insert that contained the putative initiator methionine.

5   The murine homologue of Rse was obtained by screening a murine brain cDNA library prepared in lambda gt10 (Clontech, Palo Alto CA) with a random-primed probe corresponding to nucleotides 1-1163 from the human Rse cDNA (Figure 1A). Thirteen clones were purified and the size of the inserts was determined. Two overlapping clones,

10 mbptk3.1 and mbptk3.8 (corresponding to nucleotides 737-3759 and 367-3785 of the murine Rse cDNA, respectively, of Figure 1B) were sequenced. To obtain the 5' region of the murine Rse cDNA, an oligonucleotide probe derived from the 5' end of the mbptk3.8 clone (5'-TCCAGCTACAACGCTAGCGTGGCCTGGGTGCCAGGTGCTGACGGCCTAGC (SEQ ID NO:

15 20)) was used to rescreen the murine brain cDNA library. Two positive plaques were purified, and the 5' end of the mbptk3.14 insert was sequenced and shown to contain the 5' end of the murine Rse cDNA.

    The assembled nucleotide and deduced amino acid sequences of

20 human Rse are shown in Figure 1A. The Rse cDNA sequence contains an open reading frame of 890 amino acids with two in-frame potential initiation codons (Kozak, M., J. Cell Biol. 115: 887-903 [1991]). The first of these methionine codons precedes a hydrophobic region encoding a putative signal sequence of 40 amino acids (Figures 1A

25 and 4). A second hydrophobic region is located between amino acids 429-451 and may serve as a transmembrane domain (Figure 4). This putative transmembrane region is followed by 5 basic amino acids that are characteristic of a stop transfer sequence. Thus, the mature form of human Rse is predicted to contain an ECD of 388

30 amino acids and an ICD of 439 amino acids. The human Rse cDNA was used as a basis to obtain overlapping clones encoding murine Rse cDNA from a murine brain cDNA library. The assembled nucleotide and deduced amino acid sequences are shown in Figure 1B. The murine Rse

cDNA sequence contains an open reading frame of 880 amino acids. Murine Rse contains a potential signal sequence of 30 amino acids, and a hydrophobic region between amino acids 419 and 441 that may encode a transmembrane domain (Figures 1B and 4). The overall amino acid sequence identity of murine and human Rse is 90%, with a sequence identity of 85% in the ECD and 93% in the ICD. Human and murine Rse contain significant homology in the ICD with a number of proteins. Amino acids 650-703 of murine Rse matched the partial rat Tyro-3 sequence in 54 out of 54 positions (Lai et al., *supra*); human Rse contains a single amino acid difference with rat Tyro-3; Q^{712} of human Rse is replaced with H in the rat sequence. Tyro-3 expression was detected at high levels in the rat brain, and in several other tissues that were examined. *In situ* hybridization studies show that Tyro-3 is expressed in a highly restricted pattern within the brain, with strong hybridization seen in the CA1 field but little hybridization observed in the CA2, CA3 or CA4 fields of the hippocampus (Lai et al., *supra*).

The expression of Rse in murine brain samples was also analyzed, using a probe from the ECD portion of the murine cDNA to reduce the possibility of cross-hybridization with mRNAs encoding other protein tyrosine kinases. An identical pattern of hybridization for murine Rse in the hippocampus as that previously reported for Tyro-3 was detected.

Taken together, these results indicate that Tyro-3 encodes a portion of the rat homologue of Rse. In the tyrosine kinase domain, human Rse was most similar to the human rPTKs Axl (64%), hepatocyte growth factor (HGF) receptor (45%), insulin receptor (43%), insulin-like growth factor I (IGF-I) receptor (42%) and Ros (42%) [O'Bryan, J.P., Mol. Cell. Biol. 11: 5016-5031 (1991); Janssen, J.W.G., et al., Oncogene 6:2113-2120 (1991); Park M., et al., Proc. Natl. Acad. Sci. 84: 6379-6383 (1987); Ullrich, A., et al., Nature 313: 756-761 (1985); Ullrich, A., et al., EMBO J. 5: 2503-2512 (1986); and Birchmeier, C., et al., Mol. Cell. Biol. 6: 3109-3116

(1986)]. Human and murine Rse contain a consensus site for Mg^{2+}-ATP binding ($GxGxxG(x)_{15-20}AxKxM$) beginning at amino acids 525 and 515, respectively, and a second site, IHRDLAARN (SEQ ID NO: 21), beginning at amino acids 652 and 642, respectively. These sites

5   are characteristic of protein tyrosine kinases (Hanks, et al., supra). The ECD of Rse contains 35% sequence identity with human Axl, which contains two immunoglobulin-like (IgL) repeats followed by two fibronectin type III (FNIII) repeats (Figure 4). The conserved cysteine and tryptophan residues that are characteristic

10   of IgL domains are present in human and murine Rse (Figure 4). These features indicate that Axl and Rse share a similar organization of structural domains in the ECD, and that Rse represents the second member of the Axl family of rPTKs. It is noted that Axl contains a unique sequence in the tyrosine kinase

15   domain (KWIAIE (SEQ ID NO: 22)) that has been used to distinguish it from other kinases [(K/T)W(T/M)APE (SEQ ID NO: 23)]. In this same position, Rse contains the sequence [KWLALE (SEQ ID NO: 24)] which is similar to Axl, but more similar to the HGFr (KWMALE (SEQ ID NO: 25)).

20   A distinguishing feature of the Axl/Rse family of rPTKs is the unique juxtaposition of IgL and FN-type III domains in the ECD. Axl and Rse contain two membrane distal IgL repeats and two membrane proximal FN-type III repeats. The amino acid identity of human Axl and Rse in the first and second IgL repeats is 33% and 58%

25   respectively, and 36% and 42% in first and second FN-type III domains, respectively. A similar level of amino acid identity is observed in comparison of the murine Axl and Rse IgL and FNIII domains. Without being limited to any one theory, it is believed that the combination of IgL and FNIII domains in the ECD of Rse may

30   suggest that this protein plays a role in cellular adhesion. Cell adhesion molecules are grouped into either the immunoglobulin superfamily or the cadherin family based on homology and analysis of binding properties. The cadherins mediate cell-cell adhesion in

a calcium dependent manner (Takeichi *et al.*, Annu. Rev. Biochem., **59:** 237-252 [1990]). Cadherins associate with the actin cytoskeleton through their intracellular domains via bridging proteins termed catenins (Ozawa *et al.*, EMBO J., **8:** [1989]). Cell adhesion mediated by members of the immunoglobulin superfamily is calcium-independent.

Recently, the rPTK Dtrk (Pulido *et al.*, EMBO J., **11:**391-304 [1992]), and the receptor protein phosphatase rPTPμ have been shown to promote cell adhesion in a calcium-independent homophilic manner (Brady-Kalnay *et al.*, J. Cell. Biol., **122:** 961-972 [1993]). Brady-Kalnay *et al.* have suggested that a ligand for rPTPμ may be the ECD of the same type of receptor on an adjacent cell. The interaction of the ECDs is not dependent on, nor appears to affect the properties of, the phosphatase activity of the receptor. The ECDs of human and murine Rse contain multiple consensus sites for N-linked glycosylation (NxS/T), suggesting that Rse is glycosylated (Figures 1A and 1B).

B. Construction of Cell Lines Expressing gD-Rse

To facilitate the analysis of the Rse protein, an epitope-tagged version (referred to herein as gD-Rse) was constructed. The coding sequence for the 40 amino acid signal sequence of Rse was replaced with a sequence encoding amino acids 1-53 of the herpes simplex virus type I (HSV I) glycoprotein D (gD) [Lasky, L.A., *et al.*, DNA **3:** 23-29 (1984); and Paborsky, L.R. *et al.* Pro. Eng. **3:** 547-553 (1990)]. Amino acids 1-25 encode the signal sequence of gD while amino acids 26-56 contain an epitope for the monoclonal antibody 5B6. Oligos (5'-CAGCTGCTCGAGGCAGGTCTGAAGCTCATG (SEQ ID NO: 26), and 5'-GCATGAATTCATGGCACACCTTCTACCGTG (SEQ ID NO: 27)) were used to add a Xho I site to the human Rse cDNA by PCR. The gD-Rse cDNA was inserted into the CMV-based expression vector pRK5 (Suva, L.J *et al.*, Science, **237:** 893-896 [1987]). NIH3T3 cells were transfected with the gD-Rse expression vector and the vector pCMV-

Neo using a modified CaPO4 protocol (Gorman, C., <u>DNA Cloning: A Practical Approach</u>, vol II:.143-190, Glover, D.M., ed, IRL Press, Washington D.C. [1985]). After 9 days, individual G418 resistant clones were picked and expanded.

To identify clones expressing gD-Rse, the anti-gD monoclonal antibody 5B6 was used to immunoprecipitate proteins from lysates prepared from candidate clones. Immunoprecipitates were fractionated on a 7% SDS-polyacrylamide gel under reducing conditions, and Western blots prepared from the gels were probed with the 5B6 antibody. A stable clone, 3T3.gD.R11, was isolated that expressed novel proteins of 120 kDa and 140 kDa that were reactive with 5B6 and not expressed in the parental 3T3 cells (Figure 5, lanes 1 and 2). The predicted molecular weight of gD-Rse is approximately 96 kDa. The ECD of human Rse contains 7 potential sites for N-linked glycosylation, and is glycosylated. Thus, while not being limited to any one theory, it is possible that the 120 kDa and 140 kDa forms represent different glycoforms of gD-Rse. Alternatively, the 120 kDa form may represent a proteolytically processed form of gD-Rse. Fluorescence activated cell sorting using the anti-gD monoclonal antibody 5B6 confirmed the presence of the gD epitope at the cell membrane.

C. Analysis of Tyrosine Kinase Activity of gD-Rse

The generally accepted mechanism by which ligands activate rPTKs involves ligand induced dimerization (Schlessinger, J., and Ullrich, A., <u>Neuron</u> 9: 383-391 [1992]; Ullrich, A., and Schlessinger, J., <u>Cell</u> 61: 203-212 [1990]; and Pazin, M.J., and Williams, L.T. <u>TIBS</u> 17: 374-378 [1992]). In some cases, rPTKs can be activated by antibodies directed to the receptor ECD (Yarden, Y. <u>Proc. Natl. Acad. Sci. U.S.A.</u> 87: 2569-2573 [1990]; McClain, D.A. <u>J. Biol. Chem.</u> 265: 21363-21367 [1990] and Sarup, J.C., <u>Growth Regul.</u> 1: 72-82 [1991]). It is believed that these bivalent antibodies mimic ligand-induced activation by promoting receptor

oligomerization. It was determined if an antibody (*i.e.*, monoclonal antibody 5B6) to the epitope tag of gD-Rse could function as an agonist. Serum starved 3T3.gD.R11 or control NIH3T3 cells were exposed to 5B6 monoclonal antibody, or a control antibody, for 10 minutes. Using an anti-phosphotyrosine antibody (5E2) to probe Western blots of immunoprecipitated lysates, an increase in phosphorylation of the 140 kDa form of gD-Rse in 3T3.gD.R11 cells treated with 5B6 was clearly detected (Figure 5, lanes 8 and 10). NIH3T3 cells and 3T3.gD.R11 cells were plated at a density of 2 x 10⁶ cells per 60 mm dish in DMEM:F-12 (50:50) + 10% FBS + glutamine + G418 media. After 16 hours, the media was replaced with serum-free media for 2 hours, and then antibodies were added at a concentration of 500 ng/ml. Cells were harvested, lysates were immunoprecipitated with the 5B6 antibody, fractionated by SDS-PAGE, and Western blots were probed with the indicated antibodies as described (Lokker, N.A. *et al.*, EMBO 11:2503-2510 [1992]).

As discussed below, only minor differences in phosphorylation of the 120 kDa band were observed following treatment of 3T3.gD.R11 cells with 5B6 antibody. The amount of phosphorylation of the 140 kDa band was not affected by treatment of 3T3.gD.R11 cells with control antibody (Figure 5, lane 12). As an additional control, the blots were stripped and reprobed with the 5B6 monoclonal antibody to show that the amount of gD-Rse loaded on the gel was similar (Figure 5, lanes 2, 4, and 6). As expected, the increased phosphorylation of the 140 kDa gD-Rse protein was not observed in control NIH3T3 cells treated with either the 5B6 or control antibody (Figure 5, lanes 7, 9, and 11). Thus, it was concluded that the tyrosine kinase domain of Rse is functional and that it can be regulated by receptor oligomerization. A time course experiment showed that the kinetics of antibody-induced autophosphorylation were similar to those observed with other rPTKs; induction was observed within 10 minutes, and declined gradually over the next 1-2 hours (Figure 6). These results

indicate that dimerization of the Rse receptor is sufficient to induce intrinsic tyrosine kinase activity. Considerably less antibody-induced autophosphorylation of the 120 kDa form of gD-Rse than of the 140 kDa form was detected (Figures 5 and 6). Both forms are expressed at similar levels in the 3T3.gD.R11 cells, and both contain the gD epitope. There are a number of potential explanations for this observation. For example, without being limited to any one theory, the 120 kDa gD-Rse might not be localized to the cell membrane. FACS analysis suggests that at least a portion of the gD-epitope is localized at the cell surface. However, these studies do not distinguish the relative ratios of the 120 kDa and 140 kDa forms at the membrane.

D.   Northern Analysis

The expression of Rse was characterized using Northern blot hybridization of polyadenylated RNA isolated from human tissues. A fragment from the portion of the cDNA encoding the ECD was used as a probe to minimize the possibility of cross-reaction with other tyrosine kinases. The human Rse probe was a 485 bp Pst I fragment corresponding to nucleotides 195-680 (Figure 1A). Northern blots containing 2 μg of polyadenylated RNA from various human tissues or cell lines were hybridized with random-primed probes, washed and exposed according to conditions as described by the manufacturer Clontech, Palo Alto. The RNA blot shown in Figure 7 was purchased from Clontech, Palo Alto, CA. As a control for integrity of the RNA, the blots were stripped and reprobed with a 2 Kb human b-actin DNA fragment (Clontech, Palo Alto CA). The probe detected a single predominant band of approximately 4.0 Kb (Figure 7A). The highest amount of hybridization was detected in samples of RNA from the brain and kidney, with lower expression observed in breast, heart, placenta, liver, lung, skeletal muscle, and pancreas. Probing the same blot with a control human b-actin cDNA confirmed the integrity of the RNA in all of the samples (Figure 7B). In other human

tissues that were examined, Rse was expressed at high levels in the breast and at low levels in the adrenal gland and the large and small intestine. See Table 2 below.

5

TABLE 2

Expression of Rse mRNA in Human Tissues and Cell Lines

Tissue	Expression Level[a]
Breast	+++
Adrenal	+
Large Intestine	+
Small Intestine	+
Cell Line	
CMK11-5	++
DAMI	++
THP-1	-
Hep 3B	+++
RAJI	-
K562	+
MCF 7	+
U937	+

[a]Expression quantitated as follows: (+++) = highest; (++) = moderate; (+) = weak, (-) = below limits of detection.

The expression of Rse in various human cell lines was also analyzed. Little, or no, Rse mRNA was detected by Northern blotting of mRNA samples from the monocyte cell line THP-1 or the lymphoblast-like RAJI cells (Table 2) . However, the Rse transcript was detected in a number of hematopoietic cell lines, including cells of the myeloid (i.e., myelogenous leukemia line K562 and myelomonocytic U937 cells) and the megakaryocytic leukemia lines DAMI and CMK11-5, and the human breast carcinoma cell line MCF-7.

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In the cell lines examined, the highest level of expression was observed in Hep 3B cells, a human hepatocarcinoma cell line.

E. Chromosomal Localization of Human Rse Gene

5   Primers corresponding to unique regions in the 3' end of the Rse gene were used to amplify human DNAs present in a panel of human-CHO hybrid cell lines (Figure 8). Chromosomal localization was performed using two sets of primer pairs (Btk 3-1^{2724}: 5'-CACTGAGCTGGCTGACTAAG (SEQ ID NO: 28), Btk 3-4: 5'-
10   CCTGATAGGCTGGGTACTCC (SEQ ID NO: 29); Btk 3-2^{2815}: 5'-AAGCCCGGACTGACCAAA (SEQ ID NO: 30), Btk 3-3: 5'-GTGCGGAATCAGAAAGATGG (SEQ ID NO: 31)) derived from unique sequence in the 3'-untranslated region of RSE, amplifying DNA from a panel of 25 human-hamster hybrid cell lines containing full complement of
15   the human genome (BIOS, New Haven, CT). PCR was performed with 250 ng DNA and 50 pmol each of the 5' and 3' primers, 50 mM KCl, 1.5 mM MgCl$_2$, 20 μg/ml gelatin, 0.2 mM dNTPs and 2.5 units Taq polymerase in a final volume of 100 μl. Cycles of 94°C for 30 sec, 60°C for 30 sec and 72°C for 30 sec were repeated 30 times. A portion of
20   each sample (15 μl) was electrophoresed through a 1.5% agarose gel and either visualized by ethidium bromide staining or transferred to a nylon membrane and hybridized to a ^{32}P-labeled Rse insert probe prior to 5 hour autoradiography. Positives were scored and compared to a matrix summary of human chromosomal material present
25   in each of the somatic cell hybrid, human control or hamster control DNAs. This analysis localized the Rse gene to human chromosome 15.

F. Construction of Human Rse-IgG Fusion Protein

30   The coding sequence of the ECD of Rse was fused to that of the human IgG-γ1 heavy chain in a multi-step process. PCR was used to generate a fragment with a unique BstEII site 3' to the coding sequences of the Rse amino acid 428. The 5' primer (5'-

TCAAGACAATGGAACCCA (SEQ ID NO: 32)) and the 3' primer (5'-CATGGAATTCGGTGACCGATGTGCGGCTGTGAGGAG (SEQ ID NO: 33)) were used in a 100 μl reaction containing 20 mM Tris-HCl, pH 8.2, 10 mM KCl, 6 mM $(NH_4)_2SO_4$, 1.5 mM $MgCl_2$, 0.1% Triton X-100, 200 dNTPs and 1 U of

5   Pfu DNA polymerase (Stratagene) and 50 pmol each of the forward primer and the reverse primer and 40 ng of pBS.bptk3.9, which contains a Rse cDNA insert containing most of the extracellular domain, as template. After thirty cycles of denaturation (95°C, 1 min), annealing (55°C, 30 secs) and extension (72°C, 1 min), the PCR

10   product was purified using Geneclean (Bio101), digested with BamHI and BstEII and recovered from low-melting temperature agarose gels. The PCR product was joined to the human IgG-γ_1 heavy chain cDNA through a unique BstEII site in that construct (Mark et al., J. Cell. Biol., 267: 26166-26171 [1992]). The resulting construct

15   (termed pRK.bpTK3.IgG.Fusion) contained the coding sequences for amino acids 375-428 of Rse joined to those encoding human IgG-γ_1 heavy chain. The remaining portion of the Rse ECD (amino acids 1-374) was then added by linkage through the Bam HI site in pRK.bpTK3.IgG.Fusion to yield pRK.Rse.IgG. Sequencing of the

20   construct was carried out as described above.

G. Establishment of Stable Cell Populations Expressing Rse-IgG

For stable populations, the cDNA encoding Rse-IgG was subcloned into the episomal CMV-driven expression plasmid

25   pCIS.EBON, a pRK5 derivative disclosed in Cachianes et al., Bio. Techniques, 15: 225-259 (1993), the disclosure of which is expressly incorporated herein by reference. Human fetal kidney 293 cells (obtained from ATCC, 12301 Parklawn Drive, Rockville, MD, USA) were transfected by the calcium phosphate technique. Cell

30   monolayers were incubated for four hours in the presence of the DNA precipitate, glycerol shocked, and cultured in F12:DMEM (1:1) containing 2mM glutamine, 10% fetal bovine serum, penicillin and streptomycin. After 48 hours, populations were replated in media

containing G418 to select for a stable population of cells. Conditioned media was collected from cells expressing Rse-IgG that had been cultured in serum-free media for 72 hours in the absence of G418.

H. Analysis of Rse-IgG by Western Blotting

For the Western blot analysis, 72-hour conditioned media from transfected 293 cells was subjected to electrophoresis under reducing conditions on a 7% SDS-acrylamide gel. The gel was blotted onto nitrocellulose with a Pharmacia LKB Novablot Western transfer apparatus. The filter was blocked in 1X NET (150 mM NaCl, 5 mM EDTA, 50 mM Tris-OH, pH 7.5, 0.05% Triton-X 100) with 0.25% gelatin overnight at room temperature and then incubated with an HRP-conjugated antibody to the human IgG Fc (ICN). The Western blot was developed by a chemiluminescent detection system as described by the manufacturer (Amersham).

I. Purification and Analysis of Rse-IgG

Rse-IgG was purified by affinity chromatography on a protein A column using procedures as described by Chamow, S.M., et al., Biochemistry, 29:9885-9891 (1990) with the following minor modifications. Conditioned media collected from cells expressing the Rse-IgG was adjusted to 0.1 M citrate pH 6.0 and loaded directly onto a protein A column (Repligen). The column was washed with 0.1 M citrate, pH 6.0, and was eluted with 3 M $MgCl_2$ with 10% glycerol. Fractions were pooled and desalted on a PD-10 column, dialyzed and concentrated against PBS. Protein concentrations were determined by an ELISA against human IgG (Fc). The protein was analyzed for purity by Coomassie staining of PAGE gels.

J. Generation of Rabbit Polyclonal Antisera Against Rse-IgG

Polyclonal antibodies were generated in New Zealand White rabbits against Rse-IgG. $4\mu g$ in $100\mu L$ PBS was emulsified with $100\mu L$

Freund's adjuvant (complete adjuvant for the primary injection and incomplete adjuvant for all boosts). For the primary immunization and the first boost, the protein was injected directly into the popliteal lymph nodes (Sigel et al., Methods Enzymol., 93, 3-12

5 [1983]). For subsequent boosts, the protein was injected into subcutaneous and intramuscular sites. 1.3 μg protein/kg body weight was injected every 3 weeks with bleeds taken 1 and 2 weeks following each boost.

10 K. Stimulation of 3T3.gD.R11 Cells with Anti-Rse-IgG Polyclonal Antisera

Serum starved 3T3.gD.R11 cells or NIH3T3 cells were exposed to pre-immune serum or polyclonal antisera directed against Rse-IgG at a 1/200 dilution for 10 minutes. The gD-Rse protein was

15 immunoprecipitated from extracts using the anti-gD monoclonal antibody 5B6, as described above in section B. Proteins were fractionated on a 7% SDS-PAGE under reducing conditions and transferred to nitrocellulose. Phosphoproteins were detected with the anti-phosphotyrosine antibody 5E2, as described in section C

20 above. The results are depicted in Figure 9. As can be seen in the figure, treatment of the 3T3.gD.R11 cells with anti-Rse ECD antisera stimulated the phosphorylation of the 140kDa gD-Rse protein (lane 4). This increase was not observed in cells treated with pre-immune sera.

25

L. Deposit of Materials

The following plasmid DNA has been deposited with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD, USA (ATCC):

30

Plasmid DNA	ATCC Accession No.	Deposit Date
Strain Designation "RSE"		Nov 4, 1993

This deposit was made under the provisions of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purpose of Patent Procedure and the Regulations thereunder (Budapest Treaty). This assures maintenance of a viable deposit for 30 years from the date of deposit. The plasmid DNA will be made available by ATCC under the terms of the Budapest Treaty, and subject to an agreement between Genentech, Inc. and ATCC, which assures permanent and unrestricted availability of the plasmid DNA to the public upon issuance of the pertinent U.S. patent or upon laying open to the public of any U.S. or foreign patent application, whichever comes first, and assures availability of the plasmid DNA to one determined by the U.S. Commissioner of Patents and Trademarks to be entitled thereto according to 35 USC §122 and the Commissioner's rules pursuant thereto (including 37 CFR §1.14 with particular reference to 886 OG 638).

The assignee of the present application has agreed that if the deposited DNA should be lost or destroyed when transformed into a suitable host cultivated under suitable conditions, it will be promptly replaced on notification with a specimen of the same DNA. Availability of the deposited DNA is not to be construed as a license to practice the invention in contravention of the rights granted under the authority of any government in accordance with its patent laws.

EXAMPLE 2
Isolation and Characterization of HPTK6

A.   cDNA Cloning and Sequencing

Degenerate oligodeoxyribonucleotide primers designed to sequences encoding conserved amino acids in tyrosine kinases were used to isolate a primer which was used to screen a liver carcinoma (Hep 3B) cDNA lambda library (see the procedures set

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forth in Example 1). Two full length clones encoding HPTK6 were found which differed in their 3' untranslated DNA sequences.

The assembled nucleotide and deduced amino acid sequences of human HPTK6 are shown in Figure 2. The HPTK6 cDNA sequence

5   contains an open reading frame of 913 amino acids. The mature form of human HPTK6 is predicted to contain an ECD of 417 amino acids (i.e., amino acid residues 19 to 417, shown in Figure 2) and an ICD of 473 amino acids (i.e., amino acid residues 440 to 913 shown in Figure 2). The sequence appears to be substantially

10   homologous to the human rPTK called DDR disclosed by Johnson et al., supra, sharing 99.5 % overall sequence identity therewith. Similarly, the sequence shared 93.1 % overall sequence identity with the rPTK termed NEP, which appears to be the murine equivalent of HPTK6 (see Zerlin et al., supra).

15

B.   Northern Analysis

The expression of HPTK6 was characterized via Northern blot hybridization of polyadenylated RNA isolated from human tissues. A 611 base pair fragment from the portion of the cDNA encoding

20   the ECD was used as a probe to minimize the possibility of cross-reaction with other tyrosine kinases. Northern blots containing 2 μg of polyadenylated RNA from various human tissues or cell lines were hybridized with random-primed probes, washed and exposed as described by the manufacturer Clontech, Palo Alto, CA. The RNA

25   blots shown for human adult and human fetal tissues in Figure 10 were purchased from Clontech, Palo Alto, CA. The probe detected a single predominant band of 3.8-3.9 Kb (Figure 10). In the human adult tissues, the highest amount of hybridization was detected in samples of RNA from the kidney and placenta, with lower

30   expression observed in the brain, lung, skeletal muscle and pancreas. No expression in the liver was detected. See Figure 10A. Expression of HPTK6 in the fetal tissues was different from expression in the adult tissues. With reference to Figure 10B,

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the highest expression was observed in the fetal brain with lower
expression evidenced in the fetal kidney and lung tissue,
respectively. Like the adult tissue, no expression in the liver
was observed. In the adult and fetal tissues studied, the
expression of HPTK6 was generally low.

Expression of HPTK6 in murine tissue was also investigated.
The RNA blot shown in Figures 11A and B was obtained from
Clontech, Palo Alto, CA. As a control for integrity of the RNA,
the blots were stripped and reprobed with a 2 Kb human b-actin
DNA fragment (Clontech, Palo Alto CA), Figure 11B. Bands of about
4.0 and 4.3 Kb were detected. The highest amount of hybridization
was detected in samples of RNA from the kidney and brain, with
lower expression observed in the testis, spleen and lung.

Expression of HPTK6 in various cell lines was also studied
via Northern blotting of mRNA samples from these cell lines. The
results of these experiments are shown in Table 3 below.

TABLE 3

Expression of HPTK6 mRNA in Human Cell Lines

Cell Line	Expression Level[a]
MCF 7 (human breast carcinoma)	++
Thymoma	++
Hep 3B (liver carcinoma)	+
CMK11-5 (megakaryocyte progenitor)	-
DAMI (megakaryocyte progenitor)	-
BMMC (bone marrow mononucleocytes)	-
PBMC (peripheral blood mononuclear cells)	-

Megakaryoblast   -

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* Expression quantitated as follows: (++) = highest; (+) = moderate/weak, (-) = below limits of detection.

5

In *situ* hybridization of HPTK6 RNA in deparaffinized sections of human and murine embryos was performed according to Haub & Goldfarb, *Development*, **112**: 396-406 [1991], using ^{35}S-

10   labeled cRNA riboprobes. DNA fragments from HPTK6 cDNA served as templates for synthesis of sense (+ve) and antisense (-) riboprobes. Hybridized slides were subjected to autoradiography. The traverse sections of human and mouse fetal tissues are shown in Figures 12A-C. Antisense riboprobes gave signals, while sense

15   probes gave no signal. As indicated in Figure 12, high levels of expression were observed in the fetal brain and spinal cord for both the mouse and human.

These results indicate that HPTK6 may play a role in cancer formation in certain cells, *e.g.*, human breast carcinoma cells.

20   Accordingly, antagonist ligands to the receptor may be useful for cancer therapies. The high level of expression in fetal brain indicates that HPTK6, or its ligands, may be useful for treating neurodegenerative diseases as discussed earlier herein.